The Effects of Marginal Pyridoxine Deficiency and High Protein Intakes on Vitamin B_6 Status and Enzymes in Intermediary Metabolism in Rats

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Abstract:

Pyridoxal-5-phosphate (PLP), the active form of vitamin B6 (B6), is a co-factor for enzymes in macronutrient metabolism. Increasing protein intake may affect B6 by increasing PLP-dependent enzymes in amino acid metabolism, which may be more pronounced during moderate B6 deficiency. Decreased B6 status decreases PLP-dependent enzyme activity possibly altering macronutrient metabolism. We examined changing dietary carbohydrate: protein ratios in rats consuming recommended vs. moderately deficient intakes of pyridoxine (PN)-HCl, on plasma markers of B6 status and enzymes in intermediary metabolism. Marginal B6 deficiency decreased all plasma B6 vitamers except for pyridoxic acid. Protein intake (40% energy) significantly reduced plasma PN and tended to decrease plasma pyridoxal with no significant alterations in plasma homocysteine or cysteine. Hepatic cystathionine-γ-lyase, glycogen phosphorylase, plasma aspartate and alanine aminotransferase significantly decreased with marginal B6 deficiency and cystathionine-γ-lyase decreased with increasing protein intake. Marginal B6 deficiency significantly increased hepatic glycogen with no changes in plasma haptoglobin.

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Dedication:

I dedicate this body of work to my husband Ian and mom Maria de Gloria. Thank you for being my cheerleaders here and in spirit.

Foreword

The material presented in this document has been presented in abstract form at the following conferences:

Experimental Biology, April 2015: Effect of High Protein Consumption on Markers of B_6 Status in a Rodent Model of Moderate Pyridoxine Deficiency

Canadian Nutrition Society, May 2015: Effect of High Protein Consumption on Markers of B_6 Status and PLP-Dependent Liver Enzymes in a Rodent Model of Moderate Pyridoxine Deficiency

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List of Abbreviations

A- Adequate B_6 Intake

ALT- Alanine Aminotransferase

AMDR- Acceptable Macronutrient Distribution Range

Apo-C- Apolipoprotein-C

AST- Aspartate Aminotransferase

CBS- Cystathionine Beta-Synthase

CCHS- Canadian Community Health Survey

CGL- Cystathionine Gamma-Lyase

CHO- Carbohydrate

DRI- Dietary Reference Intakes

EALT-Erythrocyte Alanine Transaminase

EAST- Erythrocyte Aspartate Transaminase

EAR- Estimated Average Requirement

EDTA- Ethylenediaminetetraacetic Acid

TG- Triglyceride

HPLC- High-Pressure Liquid Chromatography

L- Liter

M- Molar

MD- Marginal Deficient B_6 Intake

mg- Milligram

ml-Milliliter

mmol-Millimolar

MTP- Microsomal Triglyceride Protein

NADH- Nicotinamide Adenine Dinucleotide

NHANES- National Health and Nutrition Examination Survey

nmol-Nanomolar

OD- Optical Density

PN- Pyridoxine

PNP- Pyridoxine-5'-Phosphate

PA- Pyridoxic Acid

PL- Pyridoxal

PLP- Pyridoxal-5'-Phosphate

PM- Pyridoxamine

PMP- Pyridoamine-5'-Phosphate

RDA- Recommended Daily Allowance

µg- Microgram

µl- Microliter

µm- Micromolar

USA- United States of America

VLDL- Very Low-Density Lipoprotein

Introduction

Vitamin B_6 is an important water-soluble vitamin involved in carbohydrate, protein and lipid metabolism (Said, 2011). Pyridoxal-5-phosphate (PLP) is the active form of vitamin B_6 and it is a cofactor for enzymes such as cystathionine-**β**-synthase and cystathionine-**γ**-lyase (CGL) in the transsulfuration pathway. PLP is also needed for glycogen phosphorylase in the glycogenolysis pathway and aaspartate aminotransferase (AST) and alanine aminotransferase (ALT) in transamination reactions. A decrease in B_6 status (decreased plasma PLP) can decrease these PLP-dependent enzymes and impair the pathways in which they are involved. Factors lowering B_6 status include age (Morris et al., 2008; O'Leary, et al., 2011), smoking (Pfeiffer et al., 2013), inadequate intake (Institute of Medicine, 1998), food-derived inhibitors (Mayengbam, Raposo, Aliani, House, 2015), food preparation (Gregory III, 1997; Viñas et al., 2004), disease such as diabetes (Ahn, Min, & Cho 2011), hydrazine drugs (Cheng et al., 2013; di Salvo et al., 2012) and oral contraception use (Rios-Avila, et al., 2015). Vitamin B_6 status can be classified as moderate deficiency (plasma PLP 20-30 nmol/L) or severe deficiency (plasma PLP <20 nmol/L) (Institute of Medicine, 1998; Leklem, 1990). Approximately 18% of North Americans are moderately deficient, while 25% are severely deficient (Morris et al., 2008). Moderate B_6 deficiency is more commonly seen in the developed world and females and older adults seem to be at high risk (Morris et al., 2008). This can be attributed to decreased meat and overall intake as well as age, use of hydrazine drugs with age and oral contraceptives in females (Cheng et al., 2013; Lussana, Zighetti, Bucciarelli, Cugno, & Cattaneo, 2003; Garriguet, 2007)

High protein intakes in rats (70% energy as casein) decrease B_6 status by increasing B_6 requirement for protein metabolizing enzymes (Abe & Kishino, 1982; Moriya, Fukuwatari, Sano, & Shibata, 2012, Okada & Ochi, 1971; Okada et al., 1998; Pregnolato et al., 1994; Suzuki et al.,

1976; Suzuki & Okada, 1982a; Suzuki & Okada, 1982b; Suzuki & Okada, 1984). This in turn can negatively impact PLP-dependent enzymes. It should also be noted that the acceptable macronutrient distribution range (AMDR) for protein is 10- 35% of dietary energy and was set as a compliment to the already established AMDR for carbohydrates and lipid (Institute of medicine, 2008). Currently, dietary reference intakes for B_6 requirement are not considered to be influenced by protein intake (Institute of Medicine, 1998). Protein intake has been on the rise lately with the increase in popularity of high protein diets, as well as recommendations for higher protein intakes to prevent sarcopenia in older adult populations (Bauer et al., 2013; Campbell, Trappe, Wolfe, & Evans, 2001). Some of these populations consuming higher protein intakes are at risk for marginal B_6 deficiency and there is also questionable efficacy for the upper AMDR range for protein especially if one is under a marginal B_6 deficiency. Thus the extent to which protein intake near the upper AMDR range influences B_6 status measures is not clear. The further impact of moderate B_6 status in this setting is also not known. The consequences to intermediary metabolizing enzymes should be investigated.

1. Literature Review

1.1 Vitamin B_6 (B_6)

1.1.1 Properties and Structure

Vitamins are a collection of organic substances that differ in chemical structure, and need to be ingested in the diet to prevent disorders of metabolism (Pazirandeh, Lo, & Burns, 2009). Vitamin B_6 (B_6), otherwise known as pyridoxine, is a light sensitive water-soluble vitamin and consists of a group of six interchangeable pyridine compounds (also known as vitamers). They include pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL) and their 5′-phosphorylated forms (PNP, PMP and PLP), that can vary in the identity of the chemical group present at the 4′ position (Di Salvo, Contestabile, & Safo, 2011). PLP is the active form of the vitamin (Institute of Medicine, 1998; Viñas, Balsalobre, López-Erroz, & Hernández-Córdoba, 2004). PLP is an essential co-factor for over one hundred and forty enzymes in the body (Di Salvo et al., 2011), including oxidoreductases, transferases, hydrolases, lyases and isomerases, which are involved in different metabolic pathways (Percudani & Peracchi, 2003). PLP is known to participate in carbohydrate (CHO), protein, and lipid metabolism, the transsulfuration pathway, as well as the production and degradation of neurotransmitters (Allen et al., 2010; Said, 2011). PLP has a very reactive aldehyde group at the 4′ position, which accounts for its wide use as a co-factor (Di Salvo et al., 2011). Microorganisms and plants can synthesize B_6 *de novo*, while other organisms must obtain the vitamin from the diet. However, not all sources of B6 have the same bioavailability.

1.1.2 Sources & Bioavailability

Bioavailability of B_6 refers to the fraction of dietary vitamin B_6 absorbed and metabolically utilized (Nguyen & Gregory, 1983). In a mixed diet, B_6 bioavailability is

approximately 75% (Bowling, 2011). In animal tissues, B_6 is found primarily as PLP and PMP (Institute of Medicine, 1998; Viñas et al., 2004). In plants, B_6 exists as PN and PNP (Institute of Medicine, 1998), but the major proportion of B_6 (~80%) in plant proteins is found as pyridoxine glucoside (Hansen, Leklem, & Miller, 1996; Ollilainen, 1999). In rats the bioavailability of this form is reported to be 20-30% (Ink, Gregory III, & Sartain, 1986), while in humans it has been reported to not be absorbed as well as the other vitamers (Institute of Medicine, 1998). Human studies have observed decreased plasma PLP, decreased urinary end products of B_6 metabolism and a 15% loss of total B_6 intake when 27% of B_6 intake was pyridoxine glucoside versus 13% (Hansen et al., 1996). Although B_6 can also be produced from colonic bacteria (Mackey, Lieu, Carman, & Gregory III, 2003), this is minimal and requirements are met through dietary intake.

Food preparation is also known to affect B_6 bioavailability. Food processing can cause interconversion of B_6 vitamers, while leaching during cooking has been implicated as a major cause of losses (Viñas et al., 2004). Heating foods has also been seen to decrease B_6 content (Gregory III, 1997). Although the bioavailability of B_6 in foods can vary, when the vitamin is absorbed it goes through a series of processes to become biologically active.

1.1.3 Absorption and Transport

When B_6 is consumed in the diet, any phosphorylated forms of the vitamin must be hydrolyzed in the intestine by brush boarder phosphatases before absorption into enterocytes (di Salvo, Safo, & Contestabile, 2012; Merrill Jr. & Henderson, 1990). The vitamin is transported through the portal blood to the liver, where phosphorylation occurs again to ensure the vitamin does not leave the cell. This phosphorylation is done by the enzyme pyridoxal kinase, which is abundant throughout the body allowing for PL uptake at many tissues (Merrill Jr. & Henderson, 1990). In the liver, PNP and PMP are converted into PLP via pyridoxine-5 phosphate oxidase

(PNP oxidase) and pyridoxamine-5 phosphate oxidase (PMP oxidase). The majority of this enzyme is found in the liver, thus most conversions of PNP/PMP are done in this organ (Merrill Jr. & Henderson, 1990). PLP is then be bound to albumin for transport through the blood to tissues. At the tissue membrane PLP will be hydrolyzed by alkaline phosphatase for uptake into tissues and re-phosphorylated once inside to the active PLP form by PL kinase (Bowling, 2011; Masse et al. 2012). This process is known as non-saturable passive diffusion (Institute of Medicine, 1998).

1.1.4 Body Stores and Excretion

PLP and PL are the main constituents of plasma B_6 (Okada, Shibuya, Akazawa, Muya, & Murakami, 1998a), but the majority of B_6 is stored in muscle as PLP in conjunction with glycogen phosphorylase (Bowling, 2011; Okada et al., 1998a). Body storage of B_6 is reported to be around 1000 μmol (206 mg PN-HCl), with the assumption that 80% of B_6 stores are located in the muscle (Coburn et al., 1988). Stored B_6 is released from body stores at a slow rate (Lui, Lumeng, Arnoff & Li, 1985). The rate of phosphorylating PN, PM and PL to PLP by PL kinase to stay in the cell is higher than converting PNP and PMP to PL by PNP oxidase for the eventual conversion to PLP to be released from liver into circulation. This would indicate a slower release of PLP originating from PN or PM rather than PL (Merrill Jr. & Henderson, 1990). Due to slow storage release and a lack of consensus on which B_6 vitamer is the best indication of B_6 body stores, it has been difficult to accurately determine B_6 status among the population. B_6 can be measured directly through analysis of plasma, blood cells and urine, or indirectly through functional enzyme tests and methionine loading (Institute of Medicine, 1998; Miller, Nadeau, Smith, & Selhub, 1994).

B_6 is mainly excreted as PA in the urine (Hansen et al., 1996; Lui, Lumeng, Aronoff, & Li, 1985; Viñas et al., 2004), and represents around 50% of the B_6 compounds present in urine. B_6 is also found in fecal matter, although colonic bacteria production has proven difficult to measure due to the lack of precision in estimating the amount of the vitamin excreted in feces (Institute of Medicine, 1998). At present time plasma PLP alone or in combination with urinary PA, and other B_6 markers are used to assess B_6 status in humans and animals (Bowling, 2011).

1.1.5 B_6 Status Indicators

B_6 can be measured directly through analysis of plasma, blood cells and urine, or indirectly through functional enzyme tests and tryptophan or methionine loading (Institute of Medicine, 1998; Miller et al., 1994). Tryptophan loading and measurement of urinary xanthurenic acid is an indirect measurement of B_6 status. PLP is involved in tryptophan metabolism and the kynurenine pathway as a cofactor for kynureninase and kynurenine transaminase. Although xanthurenic acid has been found to be weakly associated with plasma PLP, ratios of 3-hydroxykynurenine: kynurenic acid, and kynurenic acid: xanthurenic acid have showed considerably stronger associations with PLP (Ulvik et al., 2013) and are used as an indirect measure of B_6 status.

Measuring the activity of PLP-dependent transsulfuration enzymes such cystathionine -β-synthase (CBS) and cystathionine-γ-lyase (CGL), is another indirect measure used along with the transsulfuration precursor homocysteine (HCY). Studies have shown a correlation between increased plasma HCY and decreased levels of CBS activity, CGL activity and plasma PLP concentrations, indicating depleted B_6 levels impair the transsulfuration pathway (Cabrini et al., 2005; Martinez, Cuskelly, Williamson, Toth, & Gregory, 2000). The methionine loading test evaluates B_6 status on the efficiency of PLP-dependent CBS, which is shown to bind less

strongly to PLP than CGL (Martinez et al., 2000; Miller et al., 1994). PLP-dependent serine hydroxymethyltransferase (SHMT) in the transulfuration pathway has been observed to be less tightly bound to PLP than CBS and is more readily affected during B_6 depletion (Martinez et al., 2000).

Cysteine is a product of the transsulfuration pathway and is formed from homocysteine through the intermediate cystathionine (Lima et al., 2006; Martinez et al., 2000). During B_6 deficiency an increase in plasma cystathionine and HCY are observed with decreases in plasma cysteine (da Silva et al., 2013; Lima et al., 2006; Taysi et al., 2015). Other indirect measurements include PLP-dependent enzymes such as tyrosine decarboxylase and erythrocyte alanine transaminase (EALT) and erythrocyte aspartate transaminase (EAST). The activity of these enzymes have been seen to decrease and plateau in seven to eight days, and may therefore reflect an indicator of longer term B_6 status (Hansen et al., 1996; Hansen, Shultz, Kwak, Memon, & Leklem, 2001).

PLP in tissues and plasma, and PA in urine are direct B_6 status measurements. PA excretion reflects the major portion of B_6 intake that has been absorbed and metabolized (Hansen et al., 1996). Measurement of B_6 vitamers and their metabolite, PA, in plasma have been successfully determined through electrochemical coulometry detection (Marszałł et al., 2009) and reversed phase high-pressure liquid chromatography with pre-column semicarbazide derivatives of B_6 (Talwar et al., 2003). These are more short-term B_6 status indicators as they can be readily impacted by diet.

1.2 B_6 Population Intakes and Requirements

1.2.1 B_6 Intake & Requirements

Dietary reference intakes (DRIs) are guides to help groups and individuals meet their nutritional needs to avoid deficiency and the associated risk for disease. It is recommended that groups of individuals use the estimated average requirement (EAR) to plan and assess the adequacy of nutrient intakes (Health Canada, Dietary Reference Intake Tables, 2010). The EAR is "the daily nutrient intake value that is estimated to meet the requirements, in half the apparently healthy individuals in a group" (Institute of Medicine, 2006). The recommended daily allowance (RDA) is based on the EAR values, and is used to assess individual nutrient status (Health Canada, 2010). The RDA is "the average daily intake level that is sufficient to meet the nutrient requirement of nearly all (97 to 98 percent) apparently healthy individuals in a particular life-stage and gender group" (Institute of Medicine, 2006). The EAR and RDA values for vitamin B6 for children, men and women in Canada are reported in Table 1 (Health Canada, 2010).

The values reported in Table 1 are based on healthy individuals eating a mixed North American diet. Multiple indicators of B_6 status were used to compile the data in Table 1, but as often as possible plasma PLP of at least 20 nmol/L was used as the major indicator of adequate status. Therefore plasma PLP levels below 20 nmol/L are considered B_6 deficient (Institute of Medicine, 1998). Groups consuming less than the EAR and individuals consuming less than the RDA are at risk for developing vitamin B_6 deficiency.

Table 1 Health Canada 2010 EAR & RDA Values for B_6 in Humans

Health Canada 2010 EAR & RDA Values for B_6	EAR mg/day	RDA mg/Day
Children ages: 1-3 years	0.4	0.5
Children ages: 4-8 years	0.5	0.6
Men ages: 9-13 years	0.8	1.0
Men ages: 14-50 years	1.1	1.3
Men ages: 51 years and older	1.4	1.7
Women ages: 9-13 years	0.8	1.0
Women ages: 14-18 years	1.0	1.2
Women ages: 19-50 years	1.1	1.3
Women ages: 51 years and older	1.3	1.5

Health Canada 2010 Dietary Reference Intakes Retrieved from http://www.hc-sc.gc.ca/fn-an/nutrition/reference/table/index-eng.php

1.2.2 B_6 Status in North America

Assessing B_6 status in a population can be done through analyzing food intake, measuring plasma PLP values or a combination of both. There has been a lack of data reporting large epidemiological studies on food intake in Canada and consequently it has been difficult to assess nutrient status in the Canadian population. The Canadian Community Health Survey (CCHS)-Nutrition, (2004) was the first national survey since the 1970's to analyze Canadians' eating habits. The survey involved a 24 hour diet recall and included 35,000 people across Canada, excluding individuals in the military, territories, Indian reserves, institutions and remote locations.

B_6 and its vitamers are found in protein rich muscle foods, like meat and fish, as well as nuts. These protein rich foods are classified as the meat and alternative food group in the 1992 *Canada's Food Guide to Healthy Eating for People Four Years Old and Over,* and the 2011 *Eating Well with Canada's Food Guide*. According to Garriguet (2007), in 2004, the average daily consumption of meat and alternatives in men ages 14-75 years was at least 200g. One in four men consumed more than 300 g, while all females averaged 200 g or less a day. The 2011 *Canadian Food Guide* (CFG) indicates a serving of meat as 75 grams, which is a smaller serving compared to the 1992 CFG serving of 100 grams in use at the time of the survey. The results indicate females on average met their serving requirements for meat and alternatives, while a large portion of men over consumed this food group. These findings suggest that males on average consumed more meat and alternative sources of B_6 than women, which was also observed in a nutritional survey by Starkey, Johnson-Down, & Grey-Donald (2001).

B_6 is also found in whole grains and vegetables. Although it is not known how much grain intake from the 2004 CCHS was whole grain, the amount considered a serving size stayed

the same between 1992 and 2011. Garriguet (2007) reported grain as the top energy provider for ages four and up, though with increasing age fewer grains were consumed on average. At age seventy-one and older, 43% of men and 60% of women consumed less than five servings of grain, which is less than the 1992 and 2011 CFG serving recommendations for that age group. This decreasing trend in grain consumption with age was also reported by Starkey et al. (2001). These findings indicate that with increasing age, B_6 consumption from grains decreases.

For fruits and vegetables, serving sizes have not changed since the 1992 CFG. A drawback of the 2004 CCHS is the unknown amount of vegetables versus fruit eaten in this food group. Garriguet (2007) described that seven out of ten children and half of the adults had less than five fruit and vegetable servings a day in 2004. This is less than the 1992 and 2011 CGF servings recommended for that food group. Starkey et al. (2001) also observed low intakes of fruit and vegetables among adult's years earlier, which imply that consumption of fruit and vegetables in the Canadian population is low and consequently B_6 intake from this food group is also low.

B_6 intakes from food sources in the Canadian population indicate men ages 51-70 years were 10.9% and 70 years and older were 23.1% below the EAR. Females ages 14-18 years were 11.1%, 19-30 years were 9.6%, 31-50 were 15.9%, 51-70 years were 19.4% and 70 years and older were 32.5% below the EAR (Health Canada, Canadian Community Health Survey Cycle 2.2- Nutrition, 2004).These data suggests that B_6 consumption tends to decrease with age and a small portion of females tend to have lower B_6 intakes than males. B_6 requirement increases with age so the older adult population is not meeting the increased EAR for B_6 even if consumption remained the same since early adulthood. A Nutritional study of three major Canadian Arctic cultures, which are not included in the CCHS, showed nutrient intakes were significantly and

consistently higher when at least one item of traditional food, usually meat or fish, was included in daily intake compared to non-traditional foods (Kuhnlein, Receveur, Soueida, & Berti, 2008). Increased nutrient intakes included protein and pyridoxine especially for the Inuit. In these artic cultures, 40% of dietary energy was from traditional or manufactured meats and fish and this was a leading factor in determining the adequacy for intakes of protein, minerals and vitamins such as B_6 (Kuhnlein et al., 2008).

Morris et al. (2008) conducted a large national health and nutrition examination survey (NHANES) for the United States of America (USA), and examined B_6 status in the USA from the years 2003-2004. They observed similar finding in the USA population, noting that adults between the ages of 21-44 years had low plasma PLP values, and estimated that 40% of women in this age category had low plasma PLP. Men were also seen to have declining plasma PLP with age. Although Morris et al. (2008) reported a trend for decreasing B_6 intakes with age and decreased plasma PLP in women, their results indicate teenagers had the lowest B_6 intake. This was not seen in the Canadian population; instead the elderly were seen to have the lowest B_6 status. The interesting finding from the Morris et al. (2008) study was plasma PLP was often low when B_6 intake was between 2 and 2.9 mg/d, which is well above the RDA for all age groups. NHANES data over a three-year span (2003-2006) still indicate that older adults (>60 years) have lower B_6 status compared to younger adults and women continued to have lower plasma PLP than men (Pfeiffer et al., 2013)**.** In young adults (20–39 y) 9.9% were seen to severely B_6 deficient; children and adolescents were less likely to be at risk of vitamin B_6 deficiency (<5%), and older adults were significantly more likely to be at risk (16%). This study also observed B_6 was one of the four nutrients that had the highest prevalence in persons at risk of deficiency (6–10.5%).

The 2004 CCHS analyzed B_6 intake from food, while Morris et al. (2008) analyzed intake from food and plasma PLP levels. The CCHS has limited insight on the population's dietary intakes of B_6 and its reflection on plasma PLP values. Another limitation of the 2004 CCHS is the lack of data representing the reserve-based aboriginal population who have high rates of diabetes (Health Canada, 2000), which has been associated with depleted B_6 status (Ahn, Min, & Cho, 2011).

1.3 B_6 Deficiency

1.3.1 Factors Affecting B_6 Status

B_6 status is affected by the quantity of B_6 consumed and its bioavailability. Any reduction in the availability of PLP inside the cell causes the incomplete transformation of newly formed apoenzymes into active holoenzymes in turn affecting physiological processes dependent on those enzymes (di Salvo et al., 2012; Martinez et al., 2000). Inadequate B_6 intake is observed in North America, especially in women, and it is thought to be related to decreased intakes of protein and B_6 intakes below the RDA (Gregory III, 1997). B_6 deficiency is defined as having plasma PLP levels lower than 20 nmol/L (Institute of Medicine, 1998). Severe B_6 deficiency is rare because the vitamin is found widely in diet (Marszałł et al., 2009), but as previously mentioned, the bioavailability differs. Marginal B_6 deficiency is said to be seen when plasma PLP concentration levels are 34.4 nmol/L (Rose et al., 1976), while other have suggested a range between 20-30 nmol/L (Leklem, 1990). Marginal B_6 deficiency is seen in about 18% of the population in North America and up to 50% in Europe (Haller, Lowik, Ferry, & Ferro-Luzzi, 1991; Morris et al., 2008). Health effects in marginal deficiency are usually not exhibited until much more severe deficiency occurs. PLP-dependent enzymes are widespread in many phases of metabolism; it is hard to calculate specific clinical and biochemical changes with mild deficiency

(Gregory III, 1997). Portions of the population have been reported to be deficient as a result of smoking, inadequate intake, imbalanced diet, age, drug interactions and disease (Ahn et al., 2011; Cheng et al., 2013; Morris et al., 2008; Pfeiffer et al., 2013; Rios-Avila et al., 2015)

Smoking is known to have adverse health effects and in a recent study smoking was significantly related with lower plasma PLP (29%) and PA (18%), compared with nonsmokers (Pfeiffer et al., 2013). Lower PA indicates a decreased intake is also a contributor in smokers as PA is a short term B_6 status indicator.

A few studies have implicated oral contraceptive drug usage in lowering B_6 status (Lussana, et al. 2003; Morris et al., 2008). It has been suggested that oral contraceptives alter tryptophan metabolism, a B_6-dependent metabolic pathway (Bhagavan & Brin, 1983). A study in Australia did not see this connection with B_6 (McArthur, Tang, Petocz, & Samman, 2013), but a more recent study in the U.S.A indicated significant overall differences between oral contraceptive users and non-users, regardless of B_6 status, for B_6-dependent one-carbon metabolites (HCY) and tryptophan metabolites (Rios-Avila et al., 2015). This study indicates that oral contraceptives do play a role in altering B_6 dependent pathways leading to alterations in one-carbon and tryptophan metabolites suggesting a possible lowering of B_6 status.

Drugs and natural compounds containing an amine or hydrazine functional group (ie: dopamine, penicillamine) can react with PL, PLP or pyridoxal kinase and reduce B_6 availability (di Salvo et al., 2012). Long term use of cyclooxygenase inhibitors have been observed to reduce plasma PLP in humans and non-steroidal anti-inflammatory drugs such as naproxen have been seen to decrease tissue PLP in hamster and mouse models (Cheng et al., 2013). The drug isoniazid that is used in tuberculosis treatment has been linked to decreased B_6 status, due to the fact it is part of the hydrazine-based group of anti-tuberculosis medications (Pellock, Howell,

Kendig, & Baker, 1985; Whitehouse et al., 1982). Other hydrazine drugs are commonly used for depression, respiratory, and neurological diseases and some of these compounds are listed in Table 2. Contradictive to previous studies, one study found no evidence that doses of isoniazid influenced plasma PLP, but found human immunodeficiency virus (HIV) infection did negatively impact B_6 status (Cilliers et al., 2010).

Table 2 Drugs & Natural Compounds Affecting B_6 Status

Name	Medical Use
Penicillamine	Metabolite of penicillin prescribed for rheumatoid arthritis and treatment of Wilson's Disease.
Hydralazine	Used as a secondary treatment for severe hypertension
Dopamine	Neurotransmitter used in the management of Parkinson's disease, attention deficit hyperactive disorder, drug addiction & psychosis.
Levodopa	Precursor for neurotransmitters used for management of Parkinson's Disease, encephalitis, carbon monoxide poisoning.
Isoniazid	Organic compound used in the treatment of tuberculosis.
Methylxanthines	Bronchodilators used for the management of cardio obstructive pulmonary disease (COPD), bronchiolitis and asthma.
Ginkgotoxin	Neurotoxin used to treat neuronal disorders, improve brain metabolism and peripheral blood flow.
Benzodiazepines	A psychoactive drug used in the management of anxiety, insomnia, agitation, muscle spasm, alcohol withdrawal and seizures.
Theophylline	Methylxanthine derivative used to treat COPD and other respiratory diseases
Acetylsalicylic acid	Non steroidal anti-inflammatory drug (NSAID) with an anti-platelet effect used to manage pain, fever and inflammation and prevent heart attack, stroke and blood clots.
Naproxen	NSAID used to relieve pain, inflammation and stiffness common in arthritis.

(Adapted from Cheng et al., 2013; Salvo, Safo & Contestabile, 2013)

The World Health Organization (WHO), as of 2004, recommended that vitamin B_6 be supplemented, 10 mg daily, for individuals taking Isoniazid, and under the following conditions: HIV infection, alcohol abuse, malnutrition, diabetes, and chronic liver disease in order to prevent peripheral neuropathy. These WHO recommendations are supported by evidence of connections between low B_6 status and age-related chronic diseases such as cardiovascular disease and diabetes which have been observed in humans and animals (Ahn et al., 2011; Keith et al., 2009; Martinez et al., 2000; Masse, Boudreau, Tranchant, Ouellette, & Ericson, 2012; Sakakeeny et al., 2012; Xiao et al., 2011).

Flaxseed seed has been advocated for its use in the diet as a source of alpha-linoleic acid and its beneficial effects on blood pressure (Khalesi, Irwin, & Schubert, 2015), but it contains the B_6 antagonist 1-amino-D-proline in the form of linatine which forms a hydrazine complex with the vitamin rendering it unavailable for the body (Mayengbam, Yang, Barthet, Aliani, & House, 2014). Administration of 10 mg/kg diet of 1-amino-D-proline to marginally B_6 deficient rats led to a 50% decrease in plasma PLP, a significant increase in cystathionine and HCY and a significant decrease in hepatic CGL (Mayengbam, Raposo, Aliani, & House, 2015). In humans this would be the same as consuming 25-30 g a day of flaxseed, which is suggested by some authors to reduce disease risk (Mason & Thompson, 2014; Saarinen et al., 2010). Flaxseed is one of many components common in today's food supply as it is advocated for increased health benefits, but the risk of reducing B_6 bioavailability is not widely recognized which may lead to further reductions in B_6 status in the population.

Excessive ethanol intake is known to decrease B_6 status (Gloria et al., 1997). Recent animal studies show that ethanol consumption increases the storage of B_6 resulting in less PLP to be released into the plasma (Miyazaki, Sano, Fukuwatari, & Shibata, 2011). Malnourishment

prior to chronic ethanol consumption negatively impacted B_6 status more severely than nourished subjects (Miyazaki et al., 2011) indicating alcohol-induced malnutrition as a negative factor for B_6 status (Miyazaki et al., 2011).

Older age seems to impact B_6 status negatively. A recent study reported over half of the elderly subjects admitted to hospital were B_6 deficient and B_6 status was negatively associated with the length of hospital stay. Deficiency was most prevalent in the oldest patients (O'Leary, Flood, Petocz, Allman-Farinelli, & Samman, 2011). B_6 deficiency also coincided with malnutrition seen in elderly patients and possibly results from decreased overall intake, as usually observed in elderly people (Garriguet, 2007). Morris et al. (2008) observed 22% of the USA elderly population in their epidemiological study had B_6 deficiency. Health Canada reported B_6 intake among different age groups in Canada and illustrated B_6 status tended to decrease with age (Canadian Community Health Survey- Nutrition, 2004). Older adults are known to consume supplements regularly as they do not consume sufficient amounts of many nutrients from food alone. A recent study in Austria observed 49% of community dwelling older adults ages 70-90 years used supplements and 54% of supplement users took them daily (Fabian, Bogner, Kickinger, Wagner, & Elmadfa, 2012). Fabian and colleagues observed supplement users had better B_6 status than nonusers, yet 28% of supplement users vs. 14% nonusers had marginal B_6 status and 19% vs. 38% were severely B_6 deficient. Pfeiffer et al. (2013) using data from the 2003-2006 NHANES report estimated that supplement users at any age had 79% higher PLP concentrations compared with nonusers and they still observed a prevalence of deficient plasma PLP levels in supplement users (7.8%) although it was still lower compared with nonusers (19%). The 2004 CCHS data suggest Canadian women (>51 years) had higher supplement consumption versus men (Shakur, Tarasuk, Corey, & O'Connor, 2012).

These reports suggest as we age, B_6 status and B_6-dependent enzymes are likely to decrease, especially if food intake decreases. Supplement use may help increase B_6 status but it is not always enough to correct marginal and severe deficiency in all older adults in North America.

Vitamin B_6 is the only water-soluble vitamin that has higher recommendations for people 51 years and older. The EAR is increased due to age and gender by 0.2 to 0.3 mg of B_6 derived from food a day (Institute of Medicine, 1998). As mentioned previously, protein foods are a source of B_6. Protein requirements in the elderly have been suggested at 0.8 g/kg body weight (Institute of Medicine, 2006). However, protein requirements have been suggested to increase to 1.0-1.2 g/kg body weight (Bauer et al., 2013). The international study group PROT-AGE, which consisted of European Union Geriatric Medicine Society, the International Association of Gerontology and Geriatrics-European Region, the International Association of Nutrition and Aging and the Australian and New Zealand Society for Geriatric Medicine, reviewed protein needs with aging. They proposed that, "older adults need more dietary protein than do younger adults to support good health, promote recovery from illness, and maintain functionality" (Bauer et al., 2013). A 14-week controlled diet study in ambulatory older men and women (55 to 77 years old), showed that the consumption of 0.8 g protein /kg body weight resulted in a 1.7 cm decrease in mid-thigh area, helping support claims from PROT-AGE (Campbell, Trappe, Wolfe, & Evans, 2001). Increasing protein recommendations in the elderly where B_6 status is already seen to be low regardless of B_6 intakes at or above the RDA may promote further declines in B_6 status.

Protein quality and intake are known to increase requirements for B_6. Fisher, Willis, & Haskell (1984) used amino acid mixtures similar to maize and casein to determine effects of protein quality on B_6 status in rodents. The authors observed an average decreased weight gain

and B_6 status regardless of vitamin B_6 intake in low quality protein consuming groups. This is thought to be due to the decreased efficiency of low quality protein in replacing body protein and more amino acids were metabolized for energy, which increases energy expenditure. Whole diet proteins would require further degradation to reach these steps, increasing PLP-dependent enzymes and therefore B_6 requirements in order to do so.

Protein quantity will also affect B_6 requirements. Consuming a lower protein diet compared to normal and higher protein diets (5, 19.8 & 50% of dietary energy) but with equal B_6 supplementation significantly increases B_6 bioavailability in rodents (Nguyen & Gregory, 1983). However, high intakes of animal derived protein such as beef seemed to increase the bioavailability of B_6 as reflected by PLP status (Nguyen & Gregory, 1983). Currently, the DRI for vitamin B_6 does not take protein intake into consideration (Institute of Medicine, 1998). The argument made by the Institute of Medicine for B_6 requirements not being affected by protein intake is based on 1) increased protein intake leads to tissue retention of PLP and a subsequent decrease in plasma PLP which may not necessarily mean a decrease in B_6 status (Institute of Medicine, 1998), 2) human studies investigating protein intake (12% and 20% energy) in men and women over a wide age range showed no changes in plasma PLP and erythrocyte AST (Pannemans, van den Berg, & Westerterp, 1994), 3) previous studies that investigated differing PN and protein levels on B_6 status indicators to obtain B_6 requirement in mg, adjusting for protein intake and assuming a linear relation between B_6 and protein, which is not justified (ie: there is not a linear relationship between protein intake and B_6 requirement) (Institute of Medicine, 1998). An argument against the Institute of Medicine's position is found in the evidence that shows a decrease in plasma PLP and retention in tissue PLP that is concurrent with decreases in other PLP-dependent enzymes (Bode et al., 1991). Also describing a state of

deficiency based on multiple functional enzyme activities along with decreases in plasma vitamers would be a more accurate reflection of B_6 status as B_6 is so widely spread in metabolism. For the second point, the current AMDR for protein intake are as high at 35% of energy, which was not explored in the human study mentioned. Furthermore, dietary fat was not kept constant between the different protein groups, which could shift the types of enzymes used to obtain energy (Pannemans et al., 1994). For the third point a linear approach may not be the best way to determine EAR recommendations based on protein consumption, but evidence exists that varying protein intake does increase PLP-dependent protein metabolizing enzymes and affects B_6 status via decreased PLP-dependent enzymes and enzymes involved in B_6 vitamer conversion (L. T. Miller et al., 1985; Okada, Shibuya, Akazawa, Muya, & Murakami, 1998b; Wei, 1999). High protein intakes and B_6 status are discussed later in further detail.

1.3.2 Signs & Symptoms of B_6 Deficiency

Biochemical and enzymatic markers of B_6 deficiency are seen prior to physical signs. Urinary PA responds immediately to changes in B_6 intake and is a good indicator of recent B_6 status (Lui et al., 1985). Urinary xanthurenic acid was one of the earliest markers for B_6 status as it is seen to increase as B_6 diminishes in the body. As mentioned previously, this is due to a decrease in PLP-dependent enzymes in the tryptophan metabolism pathway (Institute of Medicine, 1998). B_6 depletion decreases plasma and liver PLP, and CBS activity causing subsequent homocysteinemia, increased plasma cystathionine and liver homocysteine (Lamers, O'Rourke, Gilbert, Keeling, Matthews, Stacpoole, & Gregory III, 2009; Martinez et al., 2000; J. W. Miller et al., 1994).

Decreased AST is also seen during deficiency (Angel, 1980) resulting in the impairment of the gluconeogenesis pathway (Angel, 1980). Microcytic anemia is observed during depleted

B_6 states and results from a decrease in hemoglobin synthesis, a process that requires PLP for initiation (Institute of Medicine, 1998). Physical symptoms often manifest as seborrhaeic dermatitis, microcytic anemia, convulsions, depression and confusion (Miller et al., 1994). Bhagavan & Brin (1983) describe B_6 deficiency in five stages (Table 3). B_6 deficiency in rodents has some similarities to the clinical and biochemical markers observed in humans and outlined in Table 3. In rodents these signs include unkempt fur, pain, swelling and pink colouration around paws, snout and eyes, impaired gait, dermatitis, decreased food intake and body weight (Okada, Shibuya, Yamamoto, & Murakami, 1999; Scheer, Mackey, & Gregory, 2005).

An absence of clinical B_6 deficiency signs does not necessarily indicate normal B_6 function (Bhagavan & Brin, 1983). Thus functional enzyme tests should be a more accurate interpretation of B_6 status and can be combined with clinical assessment to determine status.

Table 3 Stages of B_6 deficiency in Humans

Measurement Type	Phase	Description
Questionnaire	Preliminary	Inadequate availability from diet, malabsorption or abnormal metabolism
Biochemical	Biochemical	Decrease in tissue B_6, urinary excretion, enzyme and co-enzyme activity
Clinical/Observational	Physiological & Behavioral	Decrease appetite, weight loss, insomnia, irritable, and fatigue
Clinical/Observational	Clinical	Physical deficiency symptoms such as dermatitis, poor weight gain
Clinical/Biochemical	Terminal	Tissue death if not corrected immediately

Adapted from Bhagavan & Brin (1983)

1.4 B_6 Involvement in Macronutrient and Energy Metabolism and Consequences of Deficiency

1.4.1 B_6 and Enzymes Linked to Intermediary Metabolism

PLP serves as a co-factor for many enzymes involved in different metabolic processes. PLP is most recognized for its involvement in the transsulfuration pathway where it acts as a cofactor for the enzymes CBS and CGL in the interconversion of HCY to the amino acid CYS (Lima et al., 2006; Martinez et al., 2000). A large population based study using non-fasting plasma samples from 10,601 healthy subjects (mean age of 56 years), found PLP had the strongest inverse correlation with HCY and cystathionine, an intermediate in the transsulfuration pathway (Midttun, Hustad, Schneede, Vollset, & Ueland, 2007), indicating that as PLP concentrations decreased there are elevations in HCY and intermediates of the transsulfuration pathways. A decrease in the transsulfuration pathway would result in decreased CYS production, which is required for the synthesis of the antioxidant glutathione (Lamers et al., 2009).

Other than its recognition in sulfur amino acid metabolism, PLP also participates in all transamination reactions and some decarboxylation and deamination reactions of amino acids (Bowling, 2011; Percudani & Peracchi, 2003). PLP functions as a cofactor for the enzyme AST, involved in the transamination reaction of aspartate and α-ketoglutarate into oxaloacetate and glutamate in gluconeogenesis (Kaimoto, Shibuya, Nlshlkawa, & Maeda, 2013; Wrenger et al., 2011). Oxaloacetate is a TCA cycle intermediate and therefore contributes to energy production, but it can also be metabolized into glucose via gluconeogenesis during fasted states. Serine dehydratase is another PLP-dependent enzyme, which deaminates serine to form pyruvate that can enter the TCA cycle (Jean et al., 2001). Transamination and decarboxylation reactions are very important for the metabolism of protein for excretion or uses in the TCA cycle (Tirapegui, Ribeiro, Pires, & Rogero, 2012) or to produce gluconeogenic amino acids (Bowling, 2011). A

recent human study indicated marginal B_6 deficiency significantly increased the ratios of plasma glutamine/glutamate and 2-oxoglutarate/glutamate (reflections of amino transaminase reactions), and tended to increase acetate and pyruvate indicating increased use of these for beta-oxidation and TCA cycle intermediates (Gregory III et al., 2013). Overt B_6 deficiency has also been shown to reduce the enzyme activities of glucose-6-phosphate dehydrogenase and malic enzyme which are required to maintain nicotinamide adenine dinucleotide phosphate (NADP) used in anabolic reactions (Angel, 1975), highlighting the significance of B_6 in energy maintenance.

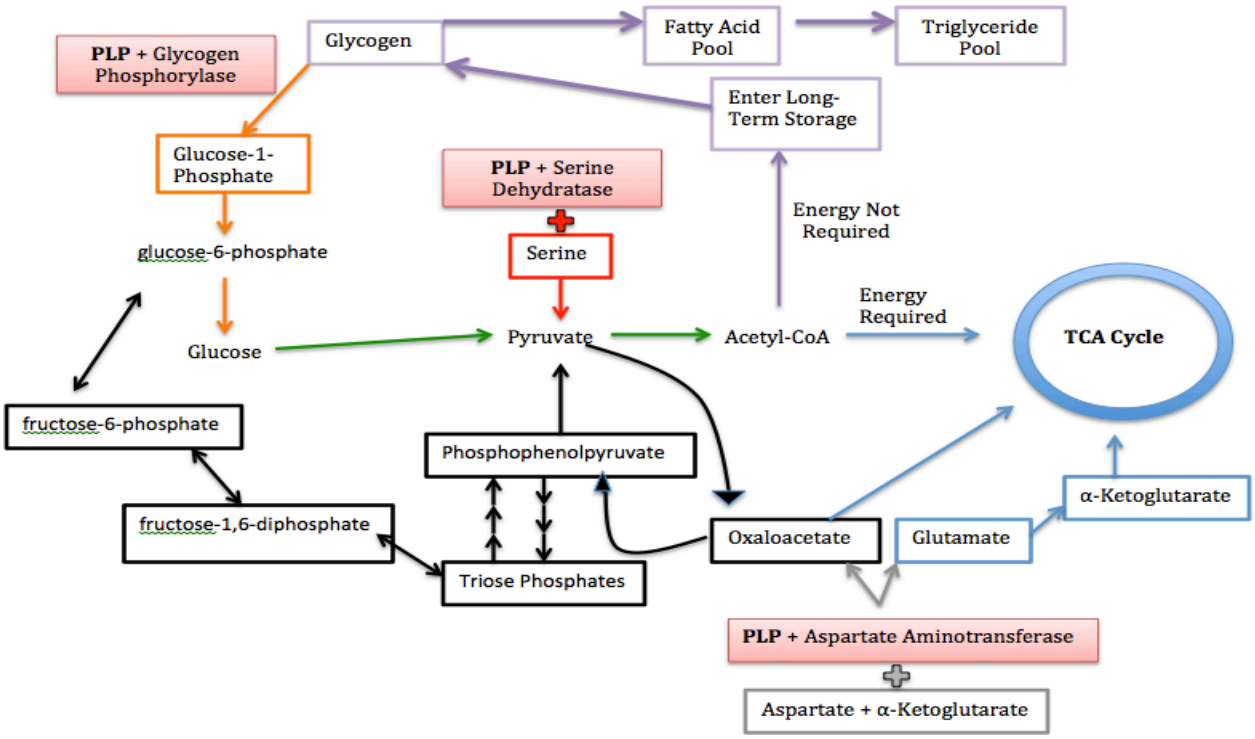
PLP is also an important cofactor in glucose metabolism. The phosphate group of PLP is an important cofactor that acts as a catalyst for the muscle enzyme glycogen phosphorylase. Glycogen phosphorylase catalyzes the first step in the glycogenolysis pathway in the fasted state, wherein glycogen is broken down to produce glucose-1-phosphate (di Salvo, Contestabile, & Safo, 2011). Glucose-1-phosphate is then converted to glucose-6-phosphate via phosphoglucomutase, to ultimately be converted into glucose. Impairments in this pathway can alter glucose utilization and force the body to use protein and lipid as carbon sources for energy production in the TCA cycle (Angel, 1975).

PLP is involved in lipid metabolism through its participation in endogenous carnitine synthesis, methylation of phospholipids and its involvement in the desaturation and elongation of fatty acids (Cho & Leklem, 1990; Hlais et al., 2012). Synthesis of carnitine, which is involved in transporting long chain fatty acids into the mitochondria for beta-oxidation to yield energy, occurs predominantly in the liver and kidneys. The biosynthesis of carnitine involves four enzymes; the second enzyme, 3-hydroxy-N-trimethyllysine aldolase requires PLP as a cofactor (Hlais et al., 2012; Vaz & Wanders, 2002). Serine transhydroxymethylase is another PLP dependent enzyme known to cleave the carnitine metabolite 3-hydroxy-6-N-trimethyllysine and

is implicated in overall carnitine biosynthesis (Dunn, Aronson, & Englard, 1982). Using a perfused liver model in rodents with depleted B_6, Dunn (1982) found that the precursors for carnitine accumulated including 3-hydroxy-6-N-trimethyllysine and less carnitine was produced. During B_6 deficiency carnitine synthesis is decreased and therefore energy metabolism is impaired (Cho & Leklem, 1990). These studies highlight the importance of B_6 for lipid transport and metabolizing enzymes in lipid metabolism.

Vitamin B_6 is an important water-soluble vitamin that acts as a cofactor for the enzymes glycogen phosphorylase, carnitine synthesis and AST involved in CHO, lipid and protein metabolism in the body. The products of these enzymes participate in maintaining blood glucose levels and energy production in the TCA cycle. Impairments in one or more of these pathways can lead to shifts in acquiring energy for the body, through shifts in intermediary metabolism.

Figure 1 B_6 Involvement in Macronutrient Metabolism



Legend: green= glycolysis, purple= glycogenesis or lipolysis, orange= glycogenolysis, black=gluconeogenesis, red= deamination, grey= transamination, blue= amino acids entering Tricarbocylic acid (TCA) cycle
Adapted from Gropper, Smith & Groff, (2009)

1.4.2. Protein Intake & its Implication on B_6 Status and Energy Regulation

The AMDR provides guidelines for allotting intake ranges of energy sources such as CHO, protein and fat, that are compatible with decreasing risks of various chronic diseases and is expressed as a percentage of energy intake (Institute of Medicine, 2006). Although the energy ranges for intakes from CHO (45-65 % energy), and fat (20-35 % energy), were based on studies that help determine decreased risk of chronic disease, the AMDR for protein (10-35 % energy) was set as a compliment to the ranges already set for CHO and fat (Institute of Medicine, 2006), making intakes at the upper AMDR for protein in reducing health risks debatable.

Healthy animals maintain glucose homeostasis over a wide AMDR range. The mechanism and signaling of insulin in skeletal muscle and adipose tissue differ during chronic intakes of different protein: CHO ratios (Devkota & Layman, 2011). Chronic higher protein intakes, around 30% energy, have been shown to decrease fasting plasma glucose and insulin secretion, as well as decrease glucose production from glycogenolysis and gluconeogenesis in humans. Moreover, gluconeogenesis was the more predominant pathway in supplying glucose to the human body (Layman, Shiue, Sather, Erickson, & Baum, 2003; Veldhorst, Westerterp-Plantenga, & Westerterp, 2009). This indicates that higher protein intakes can affect B_6-dependent enzymes, possibly glycogen phosphorylase more than AST.

Protein intakes at the upper end of AMDR range, 35% as dietary energy, have been observed to induce biphasic post-prandial insulin response in rats. This is through increased protein synthesis via muscle signaling of protein kinase B, a catalyst for protein synthesis, and decreased adipose signaling (Devkota & Layman, 2011). Although no clear reason for the biphasic response was seen, PN treatment in diabetic rats has shown PLP's role in modulating striatal insulin signaling in the forebrain through regulation of dopamine 1 and 2 receptors

(Anitha, Abraham, & Paulose, 2012). In rodents, greater insulin sensitivity without differences in plasma insulin, lower plasma glucose and better glucose tolerance has been observed. Also, increased hepatic alanine uptake, which is an important precursor for hepatic gluconeogenesis, is observed with elevated gluconeogenic enzyme activity of serine dehydratase as well as liver function enzyme ALT with high protein intakes (Blouet et al., 2006; Jean et al., 2001; Lacroix et al., 2004; Okada et al., 1998a). These studies suggest there is an inverse positive relationship with insulin and protein intakes, and high protein intakes increase protein metabolism. This in turn increases B_6 requirement for protein metabolism, which impair B_6-dependent glycogenolytic and gluconeogenic pathways. However, glycogenolysis seems to be impacted more readily (Veldhorst et al., 2009) as gluconeogenesis becomes predominant for energy metabolism and to maintain glucose homeostasis.

Increased protein intakes in rodents have been extensively documented to negatively impact B_6 status (Abe & Kishino, 1982; Okada & Ochi, 1971; Okada et al., 1998a; Pregnolato et al., 1994; Suzuki, Nakamura, Fujita, Iwami, & Abe, 1976; Suzuki & Okada, 1982a; Suzuki & Okada, 1982b; Suzuki & Okada, 1984). Intakes of 70% casein as energy in rodents were observed to suppress plasma B_6 vitamers when compared to 20% casein as energy with equivalent B_6 intake. Varying protein intakes result in different B_6 tissue saturation. Intakes of 70% casein in rodents showed tissue saturation at 5.8 mg PN-HCl/kg, while 20% casein intake reached B_6 tissue saturation at 2.9 mg PN-HCl/kg. When B_6 intake becomes inadequate, B_6 is directed toward storage in the liver and bound to cytosolic AST (Moriya, Fukuwatari, Sano, & Shibata, 2012; Okada et al., 1998a). Moriya and colleagues (2012) found after two days of starvation vitamin B_6 concentrations in the skeletal muscle decreased by 50%, and rats starved for six to nine days had 160% higher liver B_6 levels than non-starved controls. This tendency

also results when there is an increase in PLP-dependent enzyme usage as in the case of increased protein metabolism (Ito & Okada, 1973). Increased protein intake decreases B_6 status as a result of increased usage of PLP-dependent enzymes in protein amino acid metabolism. When there is an increased demand in pathways that metabolize proteins this can negatively impact B_6. Enzymes that require B_6 as a cofactor such as CBS, CGL, glycogen phosphorylase, serine deydratase and AST are all affected negatively when B_6 status is depleted. The decreased activities of these B_6-dependent enzymes impair pathways they are involved in: transsulfuration, glycogenolysis and gluconeogenesis. Depleted PLP-dependent enzymes ultimately impact the types of macronutrients used in energy metabolism, which can create shifts in the movement and storage of macronutrients.

1.4.3. Movement & Storage of Lipids During B_6 Deficiency and High Protein Intake

As highlighted in Appendix Table, multiple animal studies have shown that with increasing protein consumption, and under B_6 deficiency, liver lipids tend to increase, and enzyme activities of AST and glycogen phosphorylase decrease (Abe & Kishino, 1982; Okada & Ochi, 1971; Okada et al., 1998a; Pregnolato et al., 1994; Suzuki & Okada, 1982a; Suzuki & Okada, 1982b; Wei, 1999). Under severe B_6 deficiency, insulin is seen to promote lipogenesis, as a greater number of labeled dietary glucose ^{14}C carbons are incorporated into glycerol and free fatty acids (Angel, 1975). Without insulin the effects were similar between groups. The severe B_6 deficiency caused significant decreases in glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and acetyl CoA carboxylase in both liver and adipose tissue. The study demonstrated impairment in glycogenesis as glucose-6-phosphate dehydrogenase was inhibited, so fatty acid production was steered toward lipogenesis (Angel, 1975).

Impairments in glycogen metabolizing enzymes would indicate altered glucose utilization in B_6 deficiency. Diabetes is a disease condition in which glucose utilization is also altered by way of insulin resistance (Anitha et al., 2012) causing excess plasma glucose to be stored as lipid and energy to be obtained from protein breakdown and lipid oxidation. El-Hossary et al. (2010) used injected alloxan in rodents to induce diabetes allowing one group to go untreated (N=10) and another group to be supplemented with PN in the drinking water (2g/L) (N=10) for three months. The diabetic groups developed increased mean blood glucose, serum cholesterol and TG, but PN supplementation significantly decreased mean serum cholesterol and levels compared to the non-supplemented group. PN supplementation did not significantly alter mean blood glucose levels between diabetes groups and the PN supplemented diabetes group still had significantly higher blood glucose and serum lipids compared to controls. The decrease in serum lipids when the diabetic group was supplemented with PN may be due to increased availability of PN for glycogen storage for the enzyme glycogen phosphorylase, which would allow for excess glucose to be converted to glycogen rather than lipid. This experiment helps illustrate the significant effect B_6 has on lipid movement during periods of altered glucose utilization, which is decreased in B_6 deficiency (Angel, 1980; Okada, Ishikawa, & Watanabe, 1991; Suzuki & Okada, 1982b).

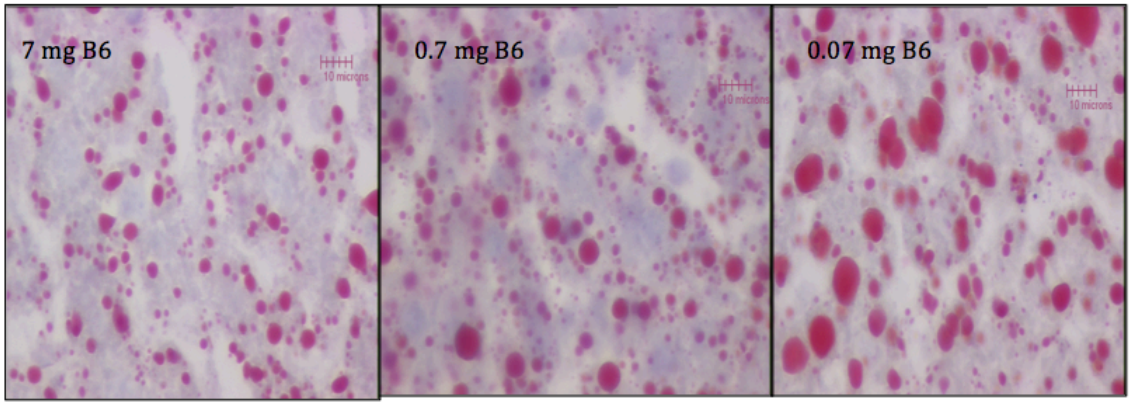
Hepatic lipid accumulation in B_6 deficiency is unique because it's induced by high protein diets whereas fatty liver induced by other conditions is often reduced by high protein diets (Suzuki & Okada, 1984). TG and cholesterol esters accumulate in liver during B_6 deficiency. The latter may be due to increased TG synthesis in liver, uptake of TG from circulation, decreased TG hydrolysis in liver, decreased fatty acid oxidation in the liver, or decreased secretion of TG from liver into circulation (Suzuki & Okada, 1984). The increase in

liver lipids during B_6 deficiency and high protein diets can translate to increases in liver weight (Abe & Kishino, 1982; Okada & Ochi, 1971; Suzuki et al., 1976) although this is not always seen (Pregnolato et al., 1994). Analysis of hepatocytes during B_6 deficiency revealed lipid droplet accumulation in hepatocytes increase with time and were also wide spread through the cytoplasm of cell (Abe & Kishino, 1982; L. W. Whitehouse, Tryphonas, & Paul, 1983). The increase in lipid droplets over time implies that the severity of fatty infiltration of the liver is associated with the length of B_6 deficiency. Lipid droplet size was seen to vary, but became smaller with time and the abnormal formation of foamy cells with small nuclei and myelin figures were observed. When PN was supplemented, liver lipids decreased and most abnormalities reversed (Abe & Kishino, 1982). Liver lipids also decreased when glucose was injected into the plasma (Suzuki & Okada, 1982a). The decrease in liver lipids and reversal of liver cell abnormalities during PN supplementation and glucose administration indicates a relationship between glucose and B_6 in the formation of liver lipids. The limitations of previous studies include the use of a model of severe deficiency, which is not seen in developed nations. We have recently documented the effects of both moderate and severe B_6 deficiency on hepatosteatosis (Raposo, unpublished data). Feeding 0.07 mg PN-HCl/kg, or 0.7 mg PN-HCl/kg dietary pyridoxine ad libitum, versus control 7 mg PN-HCl/kg diet, induced moderate and severe depleted B_6 status (Appendix Table). Decreased pyridoxine status resulted in significant hepatic lipid accumulation compared to controls, and similar hepatic lipid accumulation between the deficiency groups. (Figure 2; Raposo, unpublished data).

As mentioned above, an explanation for the increased liver lipid seen during B_6 deficiency and high protein diets is the increase in lipid movement from plasma and fat deposits into the liver as observed by Suzuki (1984) using radioactive lipid tracers. A decrease in

microsomal triglyceride transfer protein (MTP), serum very low-density lipoprotein (VLDL) and apolipoprotein-C (apo-C), TG and percent B-lipoprotein were seen. MTP, VLDL, apo-C, TG & B-lipoprotein are involved in producing lipoproteins for transport in and out of cells, and fat metabolism & transport from liver. The decreased levels of these molecules cause alterations in fat metabolism and transport, leading to the trapping of lipids in the liver. Suzuki (1982), using a rodent model of severe B_6 deficiency and high protein intake (70% casein as energy), observed elevated liver TG, cholesterol and cholesterol esters, and a decrease in serum lipoprotein, cholesterol, and cholesterol esters. This implies less TG and cholesterol being transported out of liver. An increase in radiolabeled TGs transported into the liver was also accompanied by decreases in serum VLDL, apolipoprotein-C and % B-lipoprotein, molecules responsible for lipid transport (Suzuki & Okada, 1982a; Suzuki & Okada, 1984). An inverse relationship was seen between TG and lipoproteins; as TG increased lipoproteins decreased. Therefore, more lipids are seen to move from the peripheral tissues to the visceral tissues, especially the liver, under depleted B_6 status. Synthesis of carnitine, as described earlier is involved in transporting long chain fatty acids into the mitochondria for beta-oxidation to yield energy, and B_6's indirect involvement with its synthesis creates another lipid transport dilemma when B_6 becomes inadequate (Dunn et al., 1982; Hlais et al., 2012; Vaz & Wanders, 2002). It has been observed that carnitine synthesis is decreased during B_6 deficiency resulting in impaired energy metabolism (Cho & Leklem, 1990). This coupled with increased physiological work in amino acid metabolism is a likely cause of B_6 deficient rats having impaired growth. The above results indicate that fatty degeneration of the liver during B_6 deficiency and high protein diets is due to 1) lipids being trapped in the liver, 2) a decrease in molecules associated with lipid transportation and 3) increase of TG incorporation in the liver.

Figure 2 Visualization of Hepatic Lipid Droplets Between Treatment Groups (Pilot Study)



Microphotographs of 5 µM sliced hepatic tissue from all treatment groups. Stained with Oil Red O and hematoxylin, at 40x zoom.

Rats were fed with 7, 0.7 or 0.07 mg PN-kg diet over 5 weeks. The above microphotographs are taken on liver of 5 week old Male Sprague Dawley rats.

With all this being said, hepatic lipid accumulation is not permanent. Lipid accumulation in the liver is seen when animals consumed between 40 and 70% casein and a decrease was seen when supplemented with PN or when casein was decreased (Abe & Kishino, 1982; Suzuki et al., 1976; Suzuki & Okada, 1981). PLP has also been implicated in stopping the progression of drug-induced fatty liver (Whitehouse, Boullata, & McCauley, 2008). The studies mentioned above indicate a relationship between B_6 deficiency and lipid accumulation in the liver, and their findings are summarized in Appendix Table. Limitations of these studies described here are that they are not recent and severe B_6 deficiency is induced (0 mg).

Therefore high protein intakes, under overt B_6 deficiency, can lead to disturbances in glucose metabolism, resulting in increased hepatic lipid deposition and altered energy utilization, but also result in decreases in lipid transport proteins and enzymes resulting in decreased growth and energy production.

1.4.4. Impaired Liver Integrity Under B_6 Deficiency

B_6 intake has been associated with significant decreases in molecular expression of inflammatory markers, including tumor necrosis factor (TNF)-α, interleukin (IL)-6 and cyclooxygenase-2 as well as histological markers of inflammation in a mouse model of inflammatory bowel disease (Selhub et al., 2013). In one study looking at the offspring of the Framingham study cohort, they observed that individuals with high inflammatory markers as assessed by C-reactive protein, IL-6, TNF- α, monocyte chemoattractant protein-1, fibrinogen and p-selectin, required more B_6 intake than individuals with low or normal inflammatory scores to achieve the same level of plasma PLP (Sakakeeny et al., 2012). B_6 deficiency is also indirectly associated with the decreased synthesis of the antioxidant glutathione (Lamers et al., 2009). PL has been shown to be the most efficient B_6 vitamer in reducing iron catalyzed Fenton-

mediated lipid peroxidation, protein oxidation and DNA damage in hepatocytes, while PM has been shown to reduce reactive oxygen species related damages (Mehta, Dedina, & O'Brien, 2011). During B_6 depletion the liver may be more likely to have increased oxidative damage as glutathione is decreased, which can induce an increase in chemoattractants and cytokines from hepatic Kupffer cells. Adipocytes from white adipose tissues synthesize their own catecholamines, as well as TNF- α, which contribute to increase in possible oxidative damage (Feres et al., 2013). These two processes create a perfect storm where permanent fibrotic damage can occur within the fatty liver. These studies help illustrate the concern of B_6 deficiency on hepatic health, and overall inflammation especially in a state where fatty liver may be present.

Pro-inflammatory cytokines such as TNF- α and interleukin (IL)-6 are known to have increased affinity for the liver and represent liver inflammatory markers (Yang, Lin, Yin, Albrecht, & Diehl, 1998). After inflammatory cells infiltrate the liver hepatocyte apoptosis follows, then hepatic necrosis, and elevated serum transaminases (Simpson, Lukacs, Colletti, Strieter, & Kunkel, 1997). Serum C-reactive protein has been seen as a poor marker of acute inflammation in the rat in comparison with serum haptoglobin and plasma fibrinogen, with haptoglobin being most sensitive. It is considered that haptoglobin acts as a scavenger protein functioning to conserve hemoglobin iron released from microvascular injury during a rise in inflammation (Giffen et al., 2003). Fibrinogen is synthesized in the liver in response to stimulation by IL-6 (Steel & Whitehead, 1994). Therefore either fibrinogen or haptoglobin may be good indications of inflammation in the rat.

2. Research Problems and Rationale

It has been established that PLP is an essential co-factor for numerous enzymes involved in energy and macronutrient metabolism (Selhub et al., 2013). These include: 1) transsulfuration pathway for HCY metabolism (Yamamoto, Isa, Nakagawa, & Hayakawa, 2013); 2) glycogen phosphorylase for glycogenolysis (Bode et al., 1991; Okada et al., 1991); 3) aspartate aminotransferase and serine dehydratase for gluconeogenesis (Jean et al., 2001; Kaimoto et al., 2013); and 4) all transamination and some deamination reactions of amino acid metabolism (Bowling, 2011). PLP is also involved in the methylation of phospholipids, synthesis of carnitine for lipid transport (Cho & Leklem, 1990; Hlais et al., 2012) and overall reductions in inflammation (Sakakeeny et al., 2012).

Increased protein intake decreases B_6 status as a result of increased use of PLP-dependent enzymes in protein amino acid metabolism (Ito & Okada, 1973; Okada et al., 1998a). This results in less B_6 availability for PLP-dependent enzymes in intermediary metabolism resulting in impairment in CHO, protein and lipid metabolism pathways (Angel, 1975; Miller et al., 1994; Suzuki et al., 1976; Suzuki & Okada, 1982b). Depleted PLP-dependent enzymes ultimately impact the types of macronutrients used in energy metabolism, which can create shifts in the movement and storage of macronutrients.

Under B_6 deficiency, hepatic lipid accumulation occurs and is unique because it's induced by high protein diets and other conditions that induce fatty liver are often reduced by high protein diets (Suzuki & Okada, 1982a). Hepatic steatosis is characterized by increased hepatic TG and cholesterol esters, reduced glycogenolysis and glucose utilization, and impaired hepatic lipid transport. Hepatic lipid accumulation coupled with decreased PLP-dependent synthesis of antioxidants can lead to an increased pro-oxidative state (Suzuki & Okada, 1982a;

Suzuki & Okada, 1984; Tanaka, Matsubara, Krausz, Patterson, & Gonzalez, 2012) and potentially result in increased inflammation (Raposo, unpublished data; Selhub et al., 2013). Therefore higher protein intakes can decrease B_6 status in otherwise healthy subjects and increase the potential for hepatic lipid accumulation and hepatic oxidative stress.

There is insufficient evidence to conclude health benefits of consuming protein at the upper limit of the AMDR. A recent study using rodents fed 35% energy as plant and animal whole proteins showed over 17 months had impaired renal health (Wakefield, House, Ogborn, Weiler, & Aukema, 2011). The AMDR range for protein (10-35% of dietary energy) was created to be complementary to CHO and lipid AMDR ranges already determined (Institute of Medicine, 2005). This presents the question of how differing AMDR ratios, specifically higher protein ratios, influence B_6 status, intermediary metabolism, and in turn liver integrity through the potential for increased inflammation. These effects may be accelerated in moderate B_6 deficiency, which is commonly seen in older adults, women and people using certain drugs and having certain diseases (Ahn et al., 2011; Keith et al., 2009; Martinez et al., 2000; Masse et al., 2012; Whitehouse et al., 1983; Xiao et al., 2011).

3. Research Gap

The extent to which protein intake near the upper AMDR range influences B_6 status is not clear. The further impact of moderate B_6 deficient status in this setting is also not known. Previous animal studies use older methodology, including the induction of severe B_6 deficiency and very high protein intakes, which are not applicable in the real world or seen in developed countries. The consequences to intermediary metabolizing enzymes have not been investigated.

4. Hypothesis and Objectives

Hypothesis:

High protein intake (40% as energy), in rats consuming adequate vs. marginal B_6 intakes, will decrease plasma markers of B_6 status and cause perturbations in enzymes involved in intermediary metabolism. Perturbations of intermediary metabolising enzymes may affect liver integrity by hepatic lipid accumulation and increased markers of inflammation.

Objectives:

To determine the impact of changing the ratio of carbohydrate and protein in the diet in control and moderately B_6 deficient rats on:

1) Markers of B_6 status, (plasma PLP and other B_6 vitamers, homocysteine, and cysteine, CBS and CGL),

2) Histological indices of fat accumulation and inflammation,

3) Liver injury markers (plasma AST and ALT),

4) Markers of inflammation (plasma haptoglobin), and

5) Effects on intermediary metabolism (plasma and hepatic triglycerides, total hepatic lipid, hepatic glycogen and glycogen phosphorylase).

5. Materials and Methods

5.1. Diets and Animals

Base diet formulations are listed in Table 4 and are based on the AIN-93G diet (Reeves, Nielsen, & Fahey, 1993). A two by two factorial design was employed using two levels of B_6 [marginally deficient (MD): 0.7 vs. adequate (A): 7.0 mg PN-HCL/kg diet], and two ratios of carbohydrate to protein energy: approximately 60%: 20% (AIN-93 standard; normal protein diet; 20% protein); and approximately 40%: 40% (AIN-93 modified, high protein diet; 40% energy as protein), while maintaining equal fat content. Soybean oil was the fat source used in both base diets and casein was the protein source in the in the 20% and 40% protein diets. The carbohydrate content of the 40% protein diet was decreased by 20%, with cornstarch, dextrose and sucrose contributing the same proportion of carbohydrates to the total percent energy as carbohydrate in both groups. Vitamin pre-mix was formulated to exclude pyridoxine, so that it could be added in prescribed amounts. Diet ingredients were obtained from *Dyets INC* (Pennsylvania, U.S.A.) and the diet was formulated twice throughout the experiment.

Weanling male Sprague-Dawley rats (approximately 60 grams) were received from Central Animal Care and allowed to acclimate for five days before the experiment. The rats were weighed and randomized to one of four dietary groups (n=8 per group): A-20% protein, A-40% protein, MD-20% protein, MD-40% protein for five weeks. They received food and water to permit *ad libitum* consumption and had a twelve-hour light: dark cycle. Feed intake was recorded daily by the use of weigh-backs, and weight was recorded weekly. During the experiment animal use protocols from the University of Manitoba Animal Care Committee were used in accordance with the guidelines of the Canadian Council of Animal Care (Canadian Council on Animal Care 1993).

Table 4 AIN-93G Base Mix for 20% Protein & 40% Protein Diets (% By Weight)

Ingredients	Diet Energy: 63% Carbohydrate, 20% Protein & 17% Lipid (% By Weight)	Diet Energy: 43% Carbohydrate, 40% Protein & 17% Lipid (% By Weight)
Cornstarch	39.7486	27.1143
Casein-Vitamin Free	20.0000	40.0000
Dextrose (Maltodextrin)	13.2000	9.004
Sucrose	10.0000	6.8214
Soybean Oil	7.0000	7.0000
Powdered Cellulose	5.0000	5.00
AING Mineral Mix	3.5000	3.5000
AIN 93 Vitamin Mix- No B_6	1.0000	1.0000
L-Cystine	0.3000	0.6000
Choline Bitartrate	0.2500	0.2500
T-Butylhdroquinone	0.0014	0.0014
Calories from Macronutrients (% Dietary Energy):		
Carbohydrate	63%	43%
Protein	20%	40%
Lipid	17%	17%

Both Diets listed are based on AIN-93G (Reeves et al. 1993)

Vitamin pre-mix was formulated to exclude pyridoxine, so that it could be added in prescribed amounts during the diet mixing process. Diet ingredients were obtained from *Dyets INC* (Pennsylvania, U.S.A.) and the diet was formulated twice throughout the experiment.

5.2. Plasma and Tissue Collection

Rats were terminated on day thirty-six, they were fasted over night, anesthetized with isofluorane and terminated by exsanguination via cardiac puncture. Blood was collected in heparinized vials, centrifuged at 4°C for ten minutes and plasma was collected and aliquoted into 1.5 ml microcentrifuge tubes and stored at -80°C. A portion of the liver was removed and fixed in 10% neutral buffered formalin for twenty-four hours and then moved to 70% ethanol where they were held for two weeks before being processed for histology. The remaining liver, kidneys and bicep femoris muscle were removed, weighed, quickly frozen in liquid nitrogen and stored at -80°C.

5.3. Markers of B_6 Status

5.3.1. Plasma B_6 Vitamers

Plasma B_6 Vitamers were analyzed by reverse phase high-performance liquid chromatography (HPLC) with pre-column semicarbazide derivitization (Talwar et al., 2003; Ubbink, Serfontein, & de Villiers, 1985). All standards and reagents were purchased from *Sigma Aldrich* (Ontario, Canada). A Luna C18 (2) 5 microns (250 x 4.60 mm) column (*Phenomenex*, U.S.A) was employed with a Varian 920-LC HPLC (*Varian*, U.S.A.) for analysis. All samples were prepared and analyzed under low natural light conditions, and in dark glass vials. The derivitization agent consisted of two hundred fifty mg/ml concentration of both glycine and semicarbazide. The mobile phase consisted of sixty mmol disodium hydrogen phosphate, 9.5% methanol, four hundred mL/L ethylenediaminetetraacetic acid (EDTA) disodium salt, adjusted to a pH of 6.5 and filtered through a 0.45 micron filter. Plasma, five hundred microliter (µl) was derivatized with forty µl of derivatization agent and incubated at room temperature for thirty minutes. The samples were deproteinized with 70% perchloric acid and centrifuged for ten

minutes. Then three hundred µl of supernatant was collected and stabilized with 35% sodium hydroxide. A forty µl injection volume was used for all standards and samples. The plasma vitamer concentrations were determined by comparison of the final optical density with the corresponding plasma vitamer standard curve. Plasma vitamers were reported as nanomole/liter (nmol/L).

5.3.2. Plasma HCY and CYS

Plasma total HCY and CYS concentrations were analyzed according to the reverse phase-HPLC method of Araki and Sako (1987), with modifications as suggested by Gilfix et al. (1997). All chemicals and reagents were from *Fisher Scientific* (Ontario, Canada) and *Sigma Aldrich Canada Co.* (Ontario, Canada). Plasma samples contained both protein-bound and oxidized forms of HCY, which were reduced by incubation with tris (2-carboxyethyl) phosphine. Samples were then derivatized with 7-fluorobenzofurazan-4-sulfonic acid ammonium salt. Samples and standards, forty µl, were injected into Varian 920-LC HPLC (*Varian*, U.S.A.) for separation on a Luna C18 (2) 5 microns (250 x 4.60 mm) column (*Phenomenex*, U.S.A), using an isocratic elution (98% 0.1 M acetate, pH 5.5: 2% methanol) with fluorescence detector at excitation λ = 385 nm; emission λ = 515 nm. Total HCY and CYS concentrations were determined through the use of an external standard curve. HCY and CYS were reported as micromole/liter (µm/L).

5.3.3. Hepatic CGL

Hepatic CGL activity was analyzed by colourimetric enzyme coupled kinetic assay using methods by Stipanuk & Benevenga (1997) and Stipanuk (1979). CGL catalyzes the cleavage of cystathionine to CYS, ammonia and α-ketobutyrate. This reaction oxidizes nicotinamide adenine dinucleotide (NADH), which is accompanied by a reduction in optical density at 340 nm. The rate of decrease is directly proportional to the amount of α-ketobutyrate in the sample. All

reagents, standards and chemicals were purchased from *Sigma Aldrich Canada Co.* (Ontario, Canada). A five-hundred mg frozen liver section was weighed and homogenized in ice cold 0.05 molar (M) phosphate buffer (pH 6.8) and centrifuged for thirty minutes at 4°C. The supernatant was collected and kept cold on ice. Twenty-five µl of sample was combined in a microplate with two hundred fifty µl of reaction mixture (one-hundred µl 0.1M phosphate buffer pH 7.5, fifty µl 20 mM L-cystathionine, fifty µl 1.6 mM NADH, fifty µl 7.5 U lactate dehydrogenase). Kinetic measurements were taken at 340 nm, for forty minutes at 37°C in a *BioTek Powerwave XS2* spectrophotometer (Vermont, U.S.A.). The value of the maximum slope, also known as Vmax, for each sample well is calculated by *BioTek Gen5* software. Samples were analyzed in duplicate, optimized for time and protein and were subtracted from a background reading. The optimal time was found to be forty minutes and protein concentration to be 100%. To calculate CGL activity, milli optical density was converted to optical density (OD) and the Vmax was entered into an equation, [(Vmax/min in OD)*256000nmol/L*0.275 ml]/[1000*protein (mg)], which accounted for the absorbance of NADH, the volume of the well and protein concentration. Protein was assayed by bicinchoninic acid protein assay kit (catalog number: 23225) from *Pierce/Fisher Scientific* (Ontario, Canada) as described in section 5.8. CGL is reported as nmol/minute incubation/mg protein.

5.4. Histological Staining and Grading for Inflammation

5.4.1. Histological Staining

Liver sections prepped for histology, as described in section 5.2, were taken *to Lyonel G. Israels laboratories* at the *Manitoba Tumour Bank* (Winnipeg, Canada) for embedding in paraffin wax, sectioning and staining. All products were purchased from *Fisher Scientific* (Ontario, Canada), *Mallinkrodt Pharmaceuticals* (St. Louis, U.S.A.) and *Sigma Aldrich Canada*

Co. (Ontario, Canada). Tissues were embedded in paraffin wax, cut into 5 μm sections, mounted on microscope slides and stained either for H&E or Masson's Trichome stain using in-house protocols.

Sections stained for H&E were first deparaffinised with xylene and dehydrated through descending ethanol grades. After washing in tap water, slides were immersed in Modified Harris' Hematoxylin to produce nuclei of cells deep blue. Slides were further washed in tap water and 1% acetic alcohol, ethanol and the nuclei further blued in *Scott's Tap Water* (deionized water with ammonia). Slides were counterstained by emerging slides in 0.2% Eosin to produce a pink stain in other tissues and organelles of the hepatocyte. The slide was then stained through acceding grades of ethanol and dehydrated in absolute ethanol and isopropyl and cleared with xylene.

Sections stained for *Masson's Trichrome* were first deparaffinised and dehydrated through acceding grades of ethanol, and washed in distilled water. Tissue was re-fixed in *Bouin's solution* at 56°C for one hour, and washed in water. For nuclei staining, tissues were immersed in *Weigert's iron hematoxylin* working solution and then *Biebrich scarlet-acid fuchsin solution*, for plasma staining, with water rinses in between. Slides were further immersed in phosphomolybdic-phosphotungstic acid solution to differentiate structures followed by aniline blue solution for fibrous structure staining. Water and acetic acid were used to further rinse and differentiate structures of the hepatocyte and dehydrating and clearing the stains were succeeded by immersion in absolute ethanol and xylene.

5.4.2. Histological Grading for Hepatic Injury

Paraffin embedded hepatic tissues were stained as described above in section 5.4.1and mounted on microscope slides. Sections were viewed with a Nikon Eclipse E100 microscope and

Nikon BE Plan lens 40 x/0.65 zoom (Tokyo, Japan). Pictures were taken with a *Sony* colour video camera (Tokyo, Japan). Grading for hepatic injury and steatosis were based on methods by Brunt et. al. (1999) and a protocol for obtaining pictures of the hematoxylin and eosin stained liver sections was created. First, the photographer was blinded from the treatments and then at four times and ten times magnification the entire slide was scanned by eye and using a printed documentation tool with visual aids, the photographer indicated if they could visually see central dilated veins and sinusoids, steatosis (macrovesicular and microvesicular), inflammation (focal or granulomatous), and necrosis (coagulation or liquefactions). Macrosteatosis was defined as cytoplasm being replaced by a large bubbles the fat that displaces the nucleus to the edge of the cell. Microvesicular steatosis was defined as cytoplasm being replaced by bubbles of fat but the fat does not displace the nucleus (Pouneh, Mofrad & Sanyal, 2003). Focal inflammation was defined as a single or multiple randomly distributed collection of inflammatory cells, while granulomatous was the presence of a significant number of aggregated inflammatory cells (US Department of Health and Human Services, National Toxicology Program, 1993). Coagulation necrosis was defined as some loss of cellular structure, while liquefaction necrosis was defined as a total loss of tissue structure with possible accompaniments of hemorrhage (US Department of Health and Human Services, National Toxicology Program, 1993). If any of these parameters was observed on the hepatic image a percent area was indicated. Grades were given to percent areas with grade 0 indicating <5% of the slide is observed to have that marker, grade one indicates 5-33% of the slide is observed to have that marker (mild), grade two indicates 33-66% (moderate) and grade three indicates greater than 66% observed (severe) (Brunt et al. 1999). At four times magnification a general picture of the hepatic slice with at least one central vein for a

pictorial representation was taken. If necrosis is present, *Masson's Trichrome* stained slides were viewed under the light microscope for confirmation.

5.5 Hepatic Injury and Inflammatory Makers

5.5.1. Plasma AST

AST activity was measured by colourimetric assay kit (catalog number MAK055) and 96-well clear bottom microplate from *Sigma Aldrich Canada Co.* (Ontario, Canada). Plasma AST is a catalyst for the transfer of an amino group from aspartate to α-ketoglutarate to produce oxaloacetate and glutamate. The kit uses the production of glutamate as a result of AST activity to produce a colour change, which is proportional to the AST enzyme present in the sample. Plasma, a positive control and glutamate standards were incubated at 37°C with the reaction mixture in a *BioTek Powerwave XS2* spectrophotometer (Vermont, U.S.A.) at 450 nm. After two minutes an initial absorbance reading was taken and then five more readings for each minute after until the most active sample exceeded the linear range of the standard curve. Final optical density was acquired by subtracting the last reading still within the linear range from the initial optical density reading. The plasma AST concentration was determined by comparison of the final optical density with the glutamate standard curve generated. All samples and standards were done in duplicate and were subtracted from a background reading. AST was reported as U/L.

5.5.2. Plasma ALT

A colourimetric 96-well microplate assay kit (catalog number 700260) from *Cayman Chemical* (Florida, U.S.A.) was purchased for ALT activity analysis. Plasma ALT catalyzes the transfer of an amino group from alanine to α-ketoglutarate to form pyruvate and glutamate. Pyruvate can then be metabolized to lactate via lactate dehydrogenase. This process oxidizes

NADH to NAD+. The ELISA kit monitors the conversion of NADH to oxidized NAD+ through a chromogenic agent to produce a colour change, which is associated with a decrease in absorbance at 340 nm at 37°C. The optical density is read every minute for five minutes in a *BioTek Powerwave XS2* spectrophotometer (Vermont, U.S.A.). The rate of decrease is directly proportional to the ALT activity in the plasma. The change in absorbance per minute was determined subtracting the final optical density by the initial optical density and dividing by five minutes. ALT activity (U/ml) was calculated by the equation

$(\Delta A_{340} / \min \times 0.021 \text{ ml} \div 4.11 \text{ mM}^{-1} \times 0.02 \text{ ml})$. All samples and standards were done in duplicate and were subtracted from a background reading. ALT was reported as U/L.

5.5.3. Plasma Haptoglobin

Plasma haptoglobin was analyzed via a colourimetric enzyme-linked immunosorbent assay (ELSA) kit (catalog number 2410-2) from *Life Diagnostics* (New York, U.S.A), using a *BioTek Powerwave XS2* spectrophotometer (Vermont, U.S.A.). The assay uses a 96 well microplate coated with anti-rat haptoglobin antibodies which bind the haptoglobin proteins present in plasma. After incubation for forty-five minutes, residual un-bound haptoglobin proteins were washed out of wells with water, and then incubated with horseradish peroxidase conjugated anti-haptoglobin antibodies for detection for an additional thirty minutes. The washing step was repeated and the wells were incubated with visualization agent 3,3',5,5'-Tetramethylbenzidine (TMB) to produce a blue colour due to the reaction between TMB and the bound enzyme. After twenty minutes 1 M HCl was used as a stop solution and the resulting colour was yellow. The optical density was measured at 450 nm, with the optical density of the test being relative to the concentration of haptoglobin. The concentration of plasma haptoglobin was determined from a standard curve generated from a reference standard. Samples were

diluted one hundred thousand times and this was accounted for in the final results, which are reported as ug/ml. All samples and standards were done in duplicate and were subtracted from a background reading.

5.6 Hepatic Steatosis and Lipid Movement

5.6.1. Total Hepatic Lipids

Total hepatic lipid extraction was preformed using a method by (Folch, Lees, & Sloane Stanley, 1957). Three grams of liver was homogenized for one minute, in a mixture of 2:1 chloroform/methanol for a final volume twenty times the volume of the tissue sample. The homogenate was filtered and washed with chloroform in two portions. This process was repeated once more, and water was added to the filtered solvent to create a phase separation. After the samples were allowed to sit overnight, the chloroform fraction was collected in a pre-weighed flask and evaporated using a rotary evaporator and water bath, set at 55°C. The fat content in the sample was determined by the difference in weight from the fat filled flask vs. the empty flask. Total hepatic lipids are reported as microgram/gram (μg/g) wet liver.

5.6.2. Plasma and Liver Triglycerides

Hepatic and plasma TG were analyzed by colourimetric 96-well clear bottom microplate assay kits (catalog number 10010303) from *Cayman Chemical* (Florida, U.S.A.). TG are hydrolyzed to glycerol and free fatty acids via lipoprotein lipase. The release of glycerol is measured through a series of reactions. Glycerol is phosphorylated to glycerol-3-phosphate via glycerol kinase, which is then oxidized to dihydroxyacetone phosphate and hydrogen peroxide via glycerol phosphate oxidase. A colour change occurs when hydrogen peroxide and 4-aminoantipyrine and N-Ethyl-N-(3-sulfopropyl)-*m*-anisidine are catalyzed by a redox-coupled reaction via peroxidase. The absorbance is then read at 540 nm and tissue and plasma TG were

determined from a standard curve generated from reference standards. All samples and standards were done in duplicate and subtracted from a background reading. Plasma samples were not diluted and were reported as mmol/L. For liver TG, four hundred mg of frozen liver tissue was weighed and homogenized with five ml of 50 mM sodium phosphate buffer pH 6.9 containing eighty mg/ml leupeptin. Homogenates were centrifuged for ten minutes at 4°C and supernatants were collected and placed in ice. The supernatant was diluted two times with 50 mM phosphate buffer and then analyzed as described above. Liver TGs were reported as μg/g wet liver weight.

5.7. Macronutrient Utilization

5.7.1. Total Hepatic Glycogen

Total liver glycogen was analyzed by a 96-well black bottom fluorometric assay kit (catalog number 700480) from *Cayman Chemical* (Florida, U.S.A.). Four hundred mg frozen liver tissue was weighed and homogenized with five ml of 50 mM sodium phosphate buffer pH 6.9 containing eighty mg/ml leupeptin. Homogenates were centrifuged for ten minutes at 4°C and supernatants were collected in microcentrifuge tubes and placed in ice. Supernatants were diluted twenty-five times with sodium phosphate buffer before. The kit uses amyloglucosidase to hydrolyze glycogen to form glucose, which is subsequently oxidized to hydrogen peroxide via glucose oxidase. Horseradish peroxidase was added and after incubation at 37°C with hydrogen peroxide and 10-acetyl-3,7-dihydroxyphenoxazine, the fluorescent product resorufin is formed. The fluorescence was measured using a *BioTek Synergy H4* fluorescent microplate reader (Vermont, U.S.A.) at an excitation wavelength of 530 nm and emission wavelength of 585 nm. Glycogen was determined from a generated standard reference curve. Glycogen was reported as mg/g wet liver.

5.7.2. Total Hepatic Glycogen Phosphorylase

Total hepatic glycogen phosphorylase was analyzed based on the methods of Deaciuc & Spitzer (1986) and Vilela, de Oliveira, Comar, Peralta & Bracht (2014). The assay reaction begins with the activation of glycogen phosphorylase B by phosphorylase B kinase to glycogen phosphorylase A. Glycogen phosphorylase A, in combination with a phosphate molecule, cleave an α-1-4 glucosidic bonds on the terminal end of a glycogen molecule to produce a residual glycogen chain and glucose-1-phosphate. The accumulation of inorganic phosphate from glucose-1-phosphate can be used to determine the activity of glycogen phosphorylase.

Differing incubation times and protein concentrations revealed fifteen minutes and twenty µl of hepatic supernatant (66.7% protein content) were optimal for achieving enzyme activity within the linear range of the curve. One-hundred fifty mg of frozen liver was weighed and homogenized on ice in fifteen ml centrifuge tubes with three ml of solution containing 50mM Tris buffer pH 7.4, 0.5% glycogen (w/v), 20 mM EDTA, 100 mM NaF. Homogenates were centrifuged for ten minutes at 4°C and the supernatant was collected and stored on ice. The reaction was initiated by combining three-hundred seventy µl of the reaction mixture (300 mM NaF, 50 mM glucose-1-phosphate, 1 mM 5'AMP, 500mM Na_2SO_4, 2% glycogen (w/v) at pH 6.5) with ten µl of deionized water and twenty µl of the supernatant in a microcentrifuge tube and incubating for fifteen minutes at 30°C. The reaction was then stopped and proteins were precipitated with two hundred µl of 20% (w/v) trichloroacetic acid and four hundred ul of deionized water. Another twenty µl aliquot of supernatant was also combined with ten µl deionized water and three hundred seventy µl of reaction mixture, but the reaction was stopped immediately by the addition of 20% (w/v) trichloroacetic acid and water as described above. This aliquot was used as an initial phosphate reading (sample blank). Both samples and sample

blanks were centrifuged at room temperature for ten minutes. The supernatant was collected and diluted two times with deionized water. A 96-well clear bottom colourimetric microplate assay kit purchased from *Abcam* (Massachusetts, U.S.A.) was used to measure the inorganic phosphate content of the samples and sample blanks at an absorbance of 650 nm. Total glycogen phosphorylase was calculated by standards, samples and sample blanks optical density being subtracted from the assay blank to provide a corrected optical density reading. Inorganic phosphate of the samples and sample blanks were determined from a generated standard reference curve. The inorganic phosphate content of the samples was subtracted from the sample blanks to produce the total amount of inorganic phosphate released after the reaction. This would indicate total glycogen phosphorylase activity, which was measured as the release of inorganic phosphate (nmol) per minute incubation per mg protein (nmol/min/mg protein). Protein was assayed by bicinchoninic acid protein assay kit as described in 5.8.

5.8 Total Protein Determination

The total protein concentrations of liver homogenates and plasma were determined by a bicinchoninic acid protein assay kit (catalog number: 23225) from *Pierce/Fisher Scientific* (Ontario, Canada). Liver homogenates were obtained from the analyses described above in sections 5.3.3 and 5.7.2 and diluted nine times with deionized water before assaying in a 96-well clear bottom microplate. This kit employs the biuret reaction, which uses bicinchoninic acid, the presence of peptide bonds from protein and a cupric copper ion in an alkaline medium to reduce copper to a cuprous ion. A colour change is produced from chelation of bicinchoninic acid with cuprous ion and the absorbance is linear with protein concentrations contained in the sample. Homogenates and plasma were added to the 96-well clear bottom microplate, incubated for thirty minutes at 37°C and cooled for three minutes at room temperature before assaying in a *BioTek*

Powerwave XS2 spectrophotometer (Vermont, U.S.A.). The optical density of each sample was compared to a generated standard curve from bovine serum albumin standard to determine sample protein concentration.

5.9 Statistics

SPSS software was used to perform statistics on the collected quantitative and qualitative data. Graphing the data as box-plots first identified potential outliers. Data points outside the 1.5 x interquartile range were identified as potential outliers and then trimmed from the data set if they exceeded two standard deviations away from the sample mean. Data was then analyzed for normality using the Shapiro-Wilk test statistic. Only a few dependent variables in one of the four treatment groups were identified to have a non-normal distribution, but a natural and log base 10 transformation did not normalize the data due to the small sample size (n=8). Therefore, none of the data was transformed.

A general linear model was employed with a two by two-factorial design using B_6 intake (0.7 and 7 mg PN-HCl/kg diet) and percent dietary protein energy (20% or 40%) as the independent variables. The model used a significance of $\alpha < 5\%$ to look at the main effects of B_6 and percent dietary protein energy, as well as the interaction of the two independent variables on the dependent variables. Only dependent variables with significant effects for the interaction of B_6 and percent dietary protein energy underwent a *Tukey's* Post Hoc Test.

Non-parametric Pearson's Chi Square test was used to determine significant differences between treatment groups for histological grading by level of B_6 intake or percent protein energy. A two by three contingency table with either two levels of B_6 (0.7 and 7 mg PN-HCl/kg diet), or protein level (20% or 40% protein) and three levels of grading (none, mild, or moderate) was used. The grade of severe had no data associated with it and was not included. Some cases

violated the assumption of an expected count of at least five, but the columns were not collapsed in order to prevent effects between grades from being masked. As a result the Likelihood ratio was used instead of the Pearson's Chi Square. A significance level of α <5% was used to determine significant difference between treatment groups.

The statistical software G*Power was used to determine the power of the study, using plasma B_6 as the primary outcome variable. Using a power of 0.80, in order to detect differences between four different dietary treatment groups, as well as the interaction between two B_6 levels and protein levels with a moderate effect size of 0.65 (Cohen, 1992) a sample size of n=30 was required. For analyzing the effect of protein and B_6 levels alone a sample size of n=21 was required. Therefore a total sample size of n=32 with four groups (n=8 per group) would achieve the desired power level of 0.80 for the study.

6. Results

6.1 Intakes and Growth

The adequate B_6 groups were observed to consume the most feed throughout the experiment, while the marginal B_6 groups ingested the least (Table 5). Marginal B_6 deficiency significantly decreased feed intake compared to groups with adequate B_6 status. Intakes of 40% energy as protein significantly further suppressed total feed intake and average daily intake (ADI) especially in the marginally deficient B_6 groups. No significant interaction between B_6 consumption and percent energy as protein was detected.

The adequate B_6 groups had the highest final body weights, while marginal B_6 groups had the lowest (Table 5). Marginal B_6 status significantly decreased final body weight, average daily gains (ADG) and total weight gain compared to adequate B_6 status. Intake of 40% energy as protein tended to result in lower final body weights, but only a trend was observed (p=0.07).

Total weight gain and ADG showed a significant interaction between protein and B_6 levels, which indicated the adequate and marginal B_6-40% protein groups had similar average gains. Yet, under adequate B_6 status, 40% energy as protein intake tended to result in lower weight gains while, under marginal B_6 status, the opposite was true.

Total feed conversion efficiency (FCE%) was calculated as the total weight gain divided by total intake, multiplied by one hundred. The adequate B_6-20% protein group was the most efficient at converting feed to body mass, and the marginal B_6-20% protein group the least. Marginal B_6 deficiency significantly decreased FEC%, with no significance resulting from protein intake alone. A significant interaction was observed with marginal B_6-40% protein group being as proficient at converting feed into body mass as the adequate B_6 groups.

Relative liver weights were found to be significantly decreased in marginal B_6 deficiency compared to adequate B_6 status while 40% as energy protein intake showed a trend ($P=0.07$) in decreasing relative liver weights.

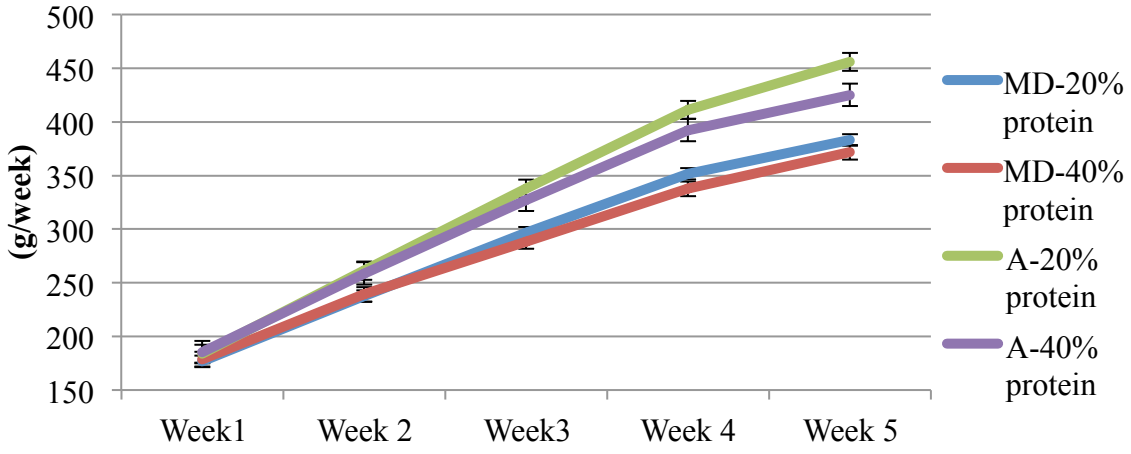
Weekly body weights (Table 6) indicated marginal B_6 status significantly decreased body weights compared to adequate B_6 status starting at week two until the end of the study. Only in the last week of the study was a decreasing trend seen for 40% as energy protein intake. For weekly intake both marginal B_6 deficiency and 40% as energy protein intake significantly reduced intake at week two until the end of the study.

Table 5 Overall Biological Performances of Marginal & Adequate B_6 Rodents with 20% & 40% Protein Intake

	0.7 mg PN/Kg Diet		7 mg PN/Kg Diet		SEM	P-Values		
	20% Protein	40% Protein	20% Protein	40% Protein		B_6 level	Protein Level	B_6 level* Protein level
Total Feed Intake (g)	805.6	773.3	961.7	882 .2	16.46	<0.001	0.002	0.17
Total ADI (g/day)	22.4	21.5	26.7	24.5	0.45	<0.001	0.002	0.17
Total Weight gain (g)	178.6^{A}	198.1^{AB}	272.2^{C}	239.8^{BC}	11.01	<0.001	0.57	0.02
Final BWT (g)	383.2	371.7	456.1	425.1	11.28	<0.001	0.07	0.39
Total ADG (g/day)	5.0^{A}	5.5^{AB}	7.6^{C}	6.7^{BC}	0.31	<0.001	0.57	0.03
Total FCE (%)	22.1^{A}	25.8^{AB}	28.4^{B}	27.2^{B}	1.26	0.005	0.31	0.06
Relative Liver Weight (g/100g bwt)	3.8	3.7	4.6	4.3	0.11	<0.001	0.07	0.39

Values are given as mean ± standard error. ADI= average daily intake, BWT= body weight, ADG= average daily gain, FCE= feed conversion efficiency= (total weight gain/total intake*100). Total Feed Intake 0.7 mg PN/Kg diet 40% protein n=7 (SEM=17.59). Total ADI 0.7 mg PN/Kg diet 40% protein n=7 (SEM=0.49). Total Weight Gain 0.7 PN/Kg diet 40% protein n=7 (SEM=11.77). ADG 0.7 PN/Kg diet 40% protein n=7 (SEM=11.77).

Figure 3 Weekly Body Weight of Marginal & Adequate B_6 Rodents with 20% & 40% Protein Intake

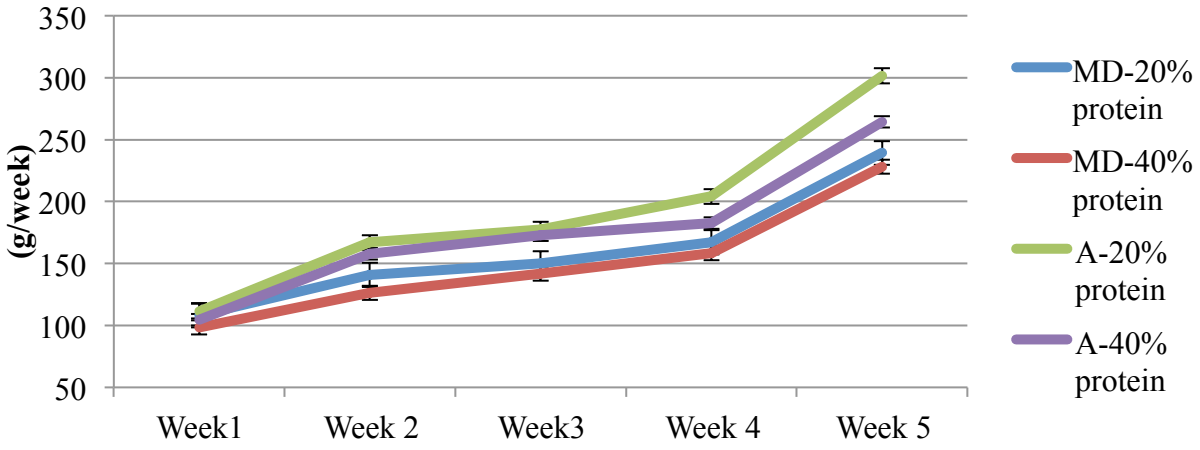


MD= marginal B_6 deficiency, A= adequate B_6

Table 6 P-Values for Weekly Body Weight of Marginal & Adequate B_6 Rodents with 20% & 40% Protein Intake

Week	B_6 level	Protein Level	B_6 level* Protein level
Week1	0.21	0.76	0.96
Week 2	0.004	0.92	0.77
Week3	<0.001	0.27	0.89
Week 4	<0.001	0.13	0.82
Week 5	<0.001	0.07	0.39

Figure 4 Weekly Intake of Marginal & Adequate B_6 Rodents with 20% & 40% Protein Intake



MD= marginal B_6 deficiency, A= adequate B_6

Table 7 P-Values for Weekly Intake for Marginal & Adequate B_6 Rodents with 20% & 40% Protein Intake

Week	B_6 level	Protein Level	B_6 level* Protein level
Week1	0.63	0.39	0.87
Week 2	<0.001	0.046	0.629
Week3	<0.001	0.027	0.765
Week 4	<0.001	0.003	0.179
Week 5	<0.001	0.053	0.284

6.2. Plasma Markers of B_6 Status

6.2.1. Plasma B_6 Vitamers

All plasma markers (PLP, PN, PL, PA) were significantly decreased under marginal B_6 deficiency (Table 8)..Under adequate B_6 status, 40% energy as protein intakes increased all plasma B_6 vitamers except for PN, which was significantly decreased compared to the 20% energy as protein. Only plasma PN was found to be significantly decreased with 40% energy as protein in both adequate and marginal B_6 status. A significant interaction between B_6 and protein levels was observed for plasma PL, indicating a significant increase in PL for the adequate B_6-40% protein group.

6.2.2. Alternative Markers of B_6 Status: Plasma HCY, CYS and Hepatic CGL

Products of the transsulfuration pathway, HCY and CYS, were not significantly altered under marginal B_6 deficiency or with protein intake (Table 9). 40% energy as protein intake under adequate B_6 status tended to decrease HCY more so than protein intake at 20% of energy, while the opposite was seen in marginal B_6 deficiency. A trend (P=0.07), was observed for the interaction between B_6 and protein levels for HCY.

Hepatic transsulfuration enzyme CGL was significantly decreased under marginal B_6 deficiency compared to adequate B_6 status. 40% energy as protein intake significantly decreased compared to the 20% energy as protein group. No interaction effect was observed between PN intake and protein levels.

6.3. Macronutrient Utilization and Enzymes Involved in Intermediary Metabolism

6.3.1. Total Hepatic Lipid

Marginal B_6 status had no effect on total hepatic lipids in comparison to adequate B_6 status but a trend (P=0.06) was observed for 40% energy as protein, which tended to further decrease hepatic lipid content (Table 10).

Table 8 Plasma B_6 Vitamers for Marginal & Adequate B_6 Rodents with 20% & 40% Protein Intake

	0.7 mg PN/Kg Diet		7 mg PN/Kg Diet		SEM	P-values		
	20% Protein	40% Protein	20% Protein	40% Protein		B_6 level	Protein Level	B_6 Level* Protein level
PA (nmol/L)	2.7	2.7	30.2	39.1	5.08	<0.001	0.47	0.47
PLP (nmol/L)	67.8	53.4	763.7	859.8	50.74	<0.001	0.43	0.29
PN (nmol/L)	181.0	90.0	235.4	192.6	20.87	0.001	0.003	0.26
PL (nmol/L)	39.3^{A}	21.6^{A}	514.8^{B}	636.2^{C}	28.16	<0.001	0.09	0.03

Values are given as mean ± standard error. PA 0.7 mg PN/Kg diet, 20% protein n=4 (SEM=7.189), PA 0.7 mg PN/Kg diet, 40% protein n=5 (SEM=6.43). PA 7.0 mg PN/Kg diet, 20% and 40% protein n=7 (SEM=30.10).

Table 9 Alternative Markers of B_6 Status for Marginal & Adequate B_6 Rodents with 20% & 40% Protein Intake

	0.7 mg PN/Kg Diet		7 mg PN/Kg Diet		SEM	P-values		
	20% Protein	40% Protein	20% Protein	40% Protein		B_6 level	Protein Level	B_6 Level* Protein level
Plasma CYS (µm/L)	280.1	286.8	281.7	272.3	7.64	0.40	0.86	0.30
Plasma HCY (µm/L)	8.5	10.7	10.1	9.6	0.79	0.70	0.30	0.09
Hepatic CGL (nmol/min/mg protein)	6.0	5.3	10.6	8.5	0.56	<0.001	0.02	0.21

Values are given as mean $\pm$ standard error.

Table 10 Plasma and Hepatic Lipid for Marginal & Adequate B_6 Rodents with 20% & 40% Protein Intake

	0.7 mg PN/Kg Diet		7 mg PN/Kg Diet		SEM	P-values		
	20% Protein	40% Protein	20% Protein	40% Protein		B_6 level	Protein Level	B_6 Level* Protein level
Plasma TG (mmol/L)	0.71	0.86	1.1	1.2	0.11	0.003	0.38	0.71
Hepatic TG (µg/g wet liver)	6.6	4.6	9.3	9.6	0.65	<0.001	0.20	0.10
Total Hepatic Lipids (mg/ g tissue)	74.7	60.7	77.1	71.8	4.88	0.176	0.06	0.376

Values are given as mean $\pm$ standard error. TG= triglyceride. Plasma TG 0.7mg PN/kg diet, 20% and 40% protein n=6 (SEM=0.12), 7mg PN/kg diet, 20 protein n=7 (SEM=1.12). Hepatic TG 0.7 mg PN/Kg diet 20% protein n=7 (SEM=6.91).

Plasma TG and hepatic TG was analyzed via colourimetric assay kits (described in section 5.51 and 5.5.2) while total hepatic lipids were analyzed via Folch method (described in section 5.6.1).

6.3.2. Plasma and Hepatic TG

Hepatic TGs were significantly decreased in moderate B_6 deficiency compared to adequate B_6 status. No other effects were observed (Table 10).

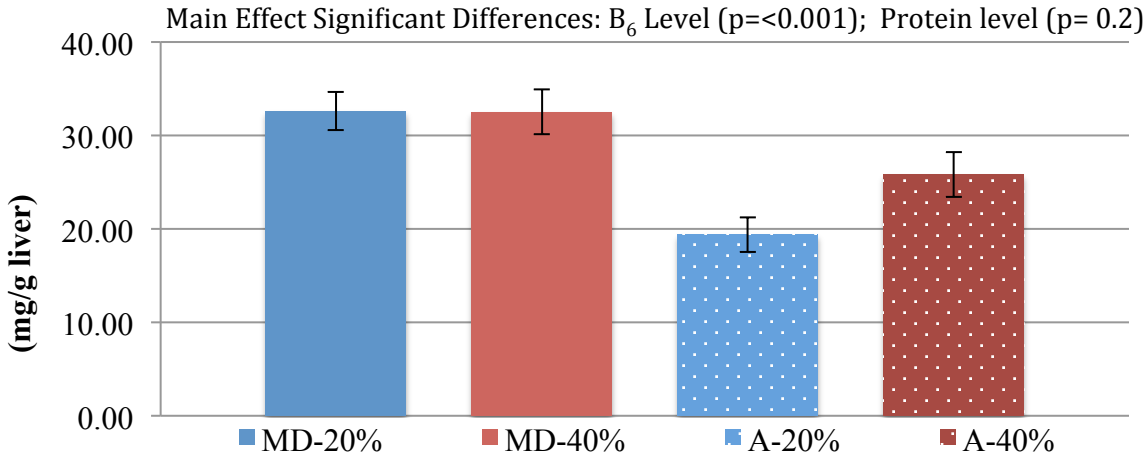
B_6 intake was the only significant effect observed for plasma TG, with the marginal B_6 deficient groups having lower plasma TG than the adequate B_6 groups.

6.3.3. Hepatic Glycogen and Glycogen Phosphorylase

Hepatic glycogen was significantly increased in marginally B_6 deficient groups compared to adequate B_6 groups (Figure 5). Protein intake had no significant effect. No significant interaction between B_6 and protein level was observed.

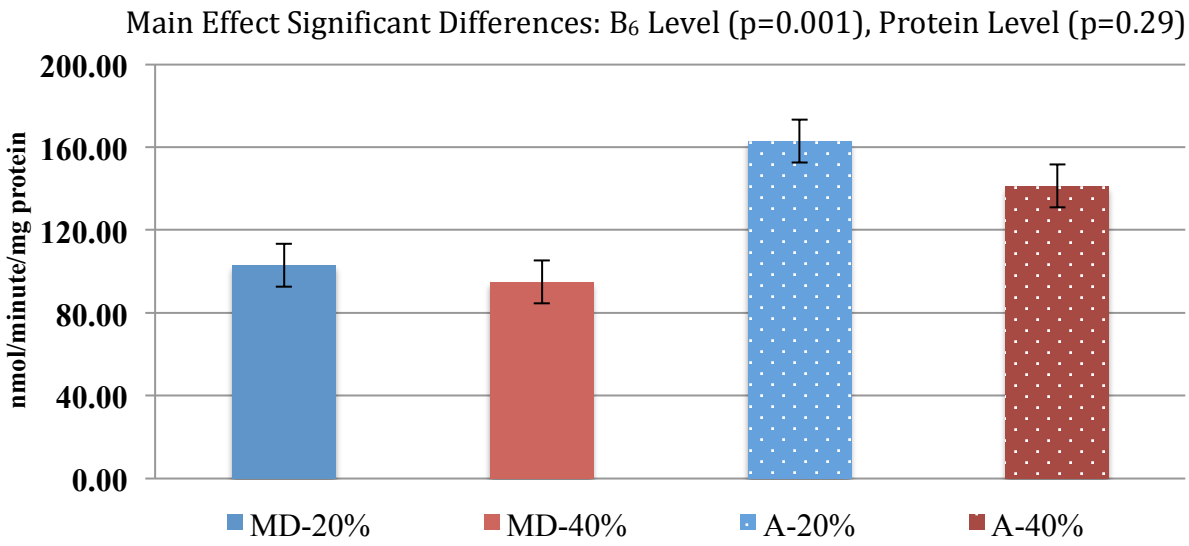
Glycogen phosphorylase was significantly decreased in the marginally deficient B_6 groups compared to adequate B_6 groups (Figure 6). No significant interaction between B_6 intake and protein level was observed.

Figure 5 Hepatic Glycogen for Marginal & Adequate B_6 Rodents with 20% & 40% Protein Intake



Values are given as mean ± standard error. MD= moderately B_6 deficient, A= adequate B_6 status. Hepatic glycogen 0.7mg PN/Kg diet, 20% n=6 (SEM=2.03), 7mg PN/Kg diet, 20% n=7 (SEM=1.88). P-values for B6*protein level 0.19.

Figure 6 Hepatic Glycogen Phosphorylase for Marginal & Adequate B_6 Rodents with 20% & 40% Protein Intake



Values are given as mean ± standard error. MD= moderately B_6 deficient, A= adequate B_6 status. Glycogen Phosphorylase 7mg PN/Kg diet, normal protein n=6 (SEM=10.38) all other groups n=7. P-values for, B_6*protein level 0.62.

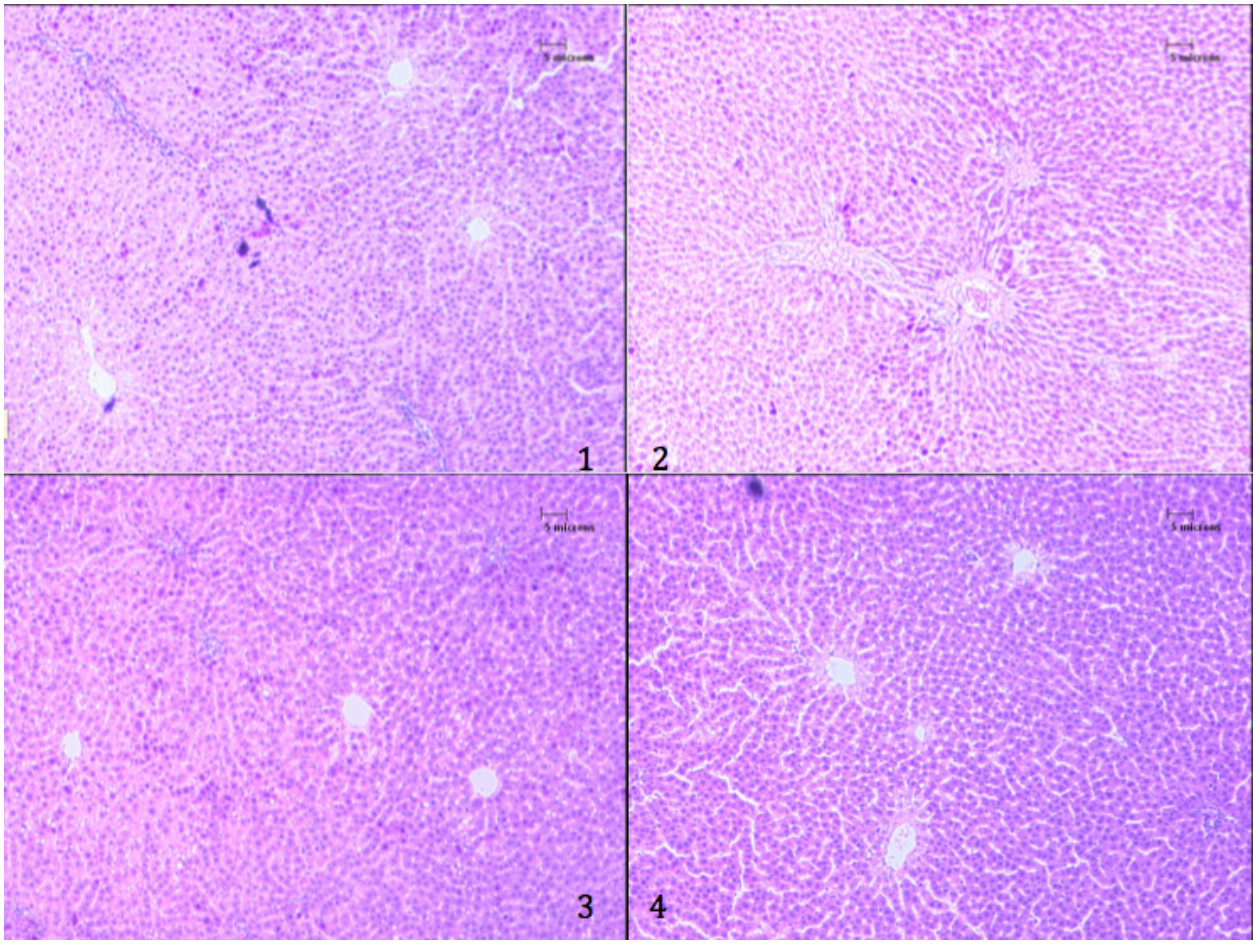
6.4. Histology for Hepatic Injury

Only Central Vein Dilation was found to be significantly different with higher protein intakes (Likelihood ratio test 7.33, N=32, P=0.026). No significant differences were seen with B_6 level. A representative histological picture from each treatment group is seen in Figure 7.

Table 11 Contingency Table for Protein Level Versus Central Vein Dilation in Rodents

Central Vein Dilation Grade:		Percent Protein 20	Percent Protein 40
None (<5%)	Count	9	2
	Expected Count	5.5	5.5
Mild (5-33%)	Count	5	11
	Expected Count	8.0	8.0
Moderate (33-66%)	Count	2	3
	Expected Count	2.5	2.5
Total	Count	16	16
	Expected Count	16.0	16.0

Figure 7 Impacts of B_6 Status and Protein Intake on Hepatic Histology of Rodents



Microphotographs of 5 **µ**M sliced hepatic tissue from all treatment groups. Stained with Hematoxylin and Eosin, 4x zoom. Groups are as follows: 1)Marginal B_6-20% protein, 2)Marginal B_6- 40% protein, 3)Adequate B_6- 20% protein, 4) adequate B_6-40% protein.

6.5. Plasma Markers of Hepatic Injury

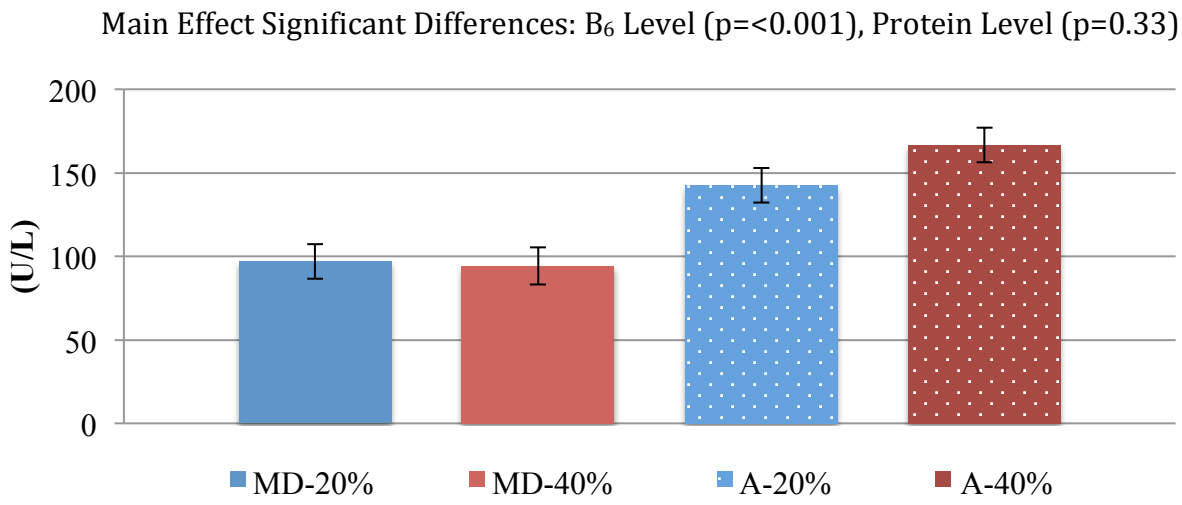
6.5.1. Plasma AST and ALT

Both plasma AST and ALT were significantly decreased under marginal B_6 deficiency compared to adequate B_6 status (Figure 8 and 9). The ALT values were around two fold higher in the adequate B_6 groups, while AST levels were around one and a half fold higher in the adequate B_6 groups. No significant interaction between B_6 intake and protein level was observed.

6.5.2. Plasma Haptoglobin

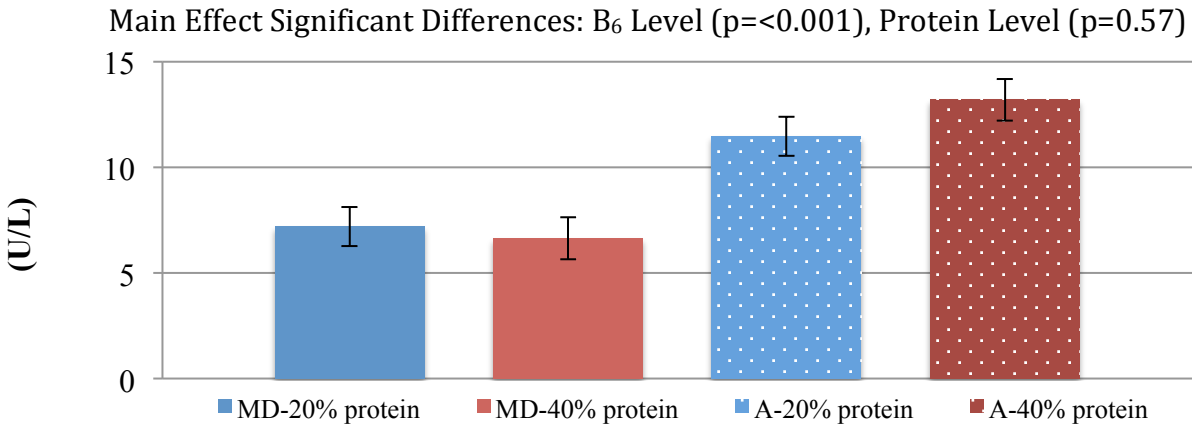
Results for plasma inflammatory marker, haptoglobin, are presented in Table 12. Results were similar across all treatments. No significant effects were seen.

Figure 8 Plasma AST for Marginal & Adequate B_6 Rodents with 20% & 40% Protein Intake



Values are given as mean ± standard error. MD= moderately B_6 deficient, A= adequate B_6 status. AST 0.7mg PN/Kg diet, 40% protein n=7 (SEM=11.12).

Figure 9 Plasma AST for Marginal & Adequate B_6 Rodents with 20% & 40% Protein Intake



Values are given as mean ± standard error. MD= moderately B_6 deficient, A= adequate B_6 status. ALT 0.7 and 7.0 mg PN/Kg diet, 40% protein n=7 (SEM=0.99).

Table 12 Plasma Haptoglobin for Marginal & Adequate B6 Rodents with 20% & 40% Protein Intake

	0.7 mg PN/Kg Diet		7 mg PN/Kg Diet		SEM	P-values		
	Normal Protein	High Protein	Normal Protein	High protein		B_6 level	Protein Level	B_6 Level* Protein level
Haptoglobin (μg/ml)	54.6	56.4	55.2	55.1	9.02	0.70	0.92	0.81

Haptoglobin 0.7mg PN/Kg diet, 40% protein n=6 (SEM=1.04), 7 mg PN/Kg diet 20% protein n=7 (SEM=0.96)

6.6. Correlations

All correlation discussed below were significant at an alpha of 0.05. Strong associations were described at r= 0.7 and higher, moderately strong associations were described as r= 0.5-0.6. weak associations were described as r= 0.3-0.4.

The strongest correlations between plasma B_6 vitamers were observed between plasma PLP, PA, and PL, which were positively associated (See Appendix). Plasma PN had positive weak or moderately strong associations between the other plasma vitamers with plasma PA showing the strongest positive association with PN. Plasma B_6 vitamers had a moderately strong, positive relationship with weight gain, intake, relative liver weight and final body weight. Plasma PLP had the strongest positive relationship with hepatic CGL, while hepatic TG had the strongest positive association with plasma PL. Plasma TG had weak positive associations with plasma B_6 vitamers, while hepatic glycogen had weak negative relationships, with the exception of PA, which had almost no relationship with glycogen. Glycogen phosphorylase had weak positive associations with plasma PLP, PA and PL. Plasma AST and ALT had strong positive associations with plasma PLP and PL, and moderate associations with PA.

Average daily intake had strong positive associations with final weight, average daily gain, relative liver weight and hepatic CGL, while moderately strong positive associations were seen with hepatic TG, plasma ALT, hepatic glycogen phosphorylase. Relative liver weight was observed to have moderately strong positive relationships with hepatic and plasma TG and hepatic CGL, while a moderately strong negative association was observed with hepatic glycogen.

Interestingly, hepatic CGL had the strongest positive association with plasma ALT followed by plasma PLP. Moderately strong and weak positive associations were also seen with

hepatic CGL and plasma and hepatic TG, plasma AST and glycogen phosphorylase activity. Hepatic TGs were weakly positively associated with most variables including plasma TG for which the same was true. Hepatic glycogen had a negative association with all variables and the strongest association was with average daily gain, followed by average daily intake, which was a moderately strong association.

Plasma AST and ALT tended to differ in their associations with other variables except for plasma B_6 vitamers, for which associations tended to be similar. The association between plasma AST and ALT was positive and moderately strong. Glycogen phosphorylase had a very weak negative association with hepatic glycogen. It should be noted that both groups had data points near the two standard deviation away from the mean, which was the criteria for determining an outlier, but was still within the acceptable range to be kept in the data. This may explain the poor association observed between the two variables.

7. Discussion

Common B_6 deficiency signs in rodents, such as unkempt fur and decreased intake and body weight (Okada et al., 1999; Scheer et al., 2005) were observed in the marginal B_6 deficient group. The decrease in body weight was significant which was not observed in our pilot study establishing moderate B_6 deficiency, indicating the rodents may have been slightly more deficient than the pilot study (Raposo, unpublished data). However all other parameters (no elevations in plasma HCY and CYS, and significant decreased hepatic B_6 vitamers) were met, indicating a marginal deficiency was reached.

In the present study, 40% energy as protein showed a trend ($p=0.07$) towards decreasing body weight while marginal B_6 deficiency significantly decreased body weight. Devkota & Layman (2011) observed a non-significant difference for body weights in their rodents consuming a similar dietary macronutrient profile (35% energy as protein, 35% energy as carbohydrate, 30% energy as lipid versus 12% energy as protein, 60% energy as carbohydrate, 30% energy as lipid) under adequate B_6 status. The mean final body weights after ten days were 275.7 g ± 1.3g and 273.7 g ± 1.6 g for the 35% and 12% energy as protein groups respectively, and they were comparable to the body weights of the adequate B_6 groups on week two of the present study. However, Devkota and Layman (2011) mimicked human feeding schedules by providing food three times a day, while the present study free access to food. The mode of feeding (free access versus meal-fed) under overt B_6 deficiency has been observed to decrease the percentage of body fat and increase percentage body water (Angel & Song, 1973). This is thought to be due to impaired fatty acid synthesis and impaired amino acid metabolism by decreasing PLP-dependent transamination and decarboxylation activities and therefore decreasing the carbon required for gluconeogenesis and energy production (Angel & Song,

1973). A decrease in ^{14}C labelled glucose being incorporated into fatty acids of the liver and carcass of rodents is observed under B_6 deficiency along with decreased activity of glucose-6-phosphatase dehydrogenase and malic enzyme responsible for NADPH maintenance (Angel & Song, 1973). The present study did observe decreased transamination reactions (plasma AST and ALT) and a decrease in total body weight in the marginal B_6 groups, so this may be due to higher amounts of dietary glucose being used as energy instead of fat storage. The mode of feeding may also be a reason as to why there was a trend for decreasing body weight with increasing protein intake in the current study in comparison to Devkota & Layman (2011). Morens et al. (2011), however, used a three meal feeding approach to administer isocaloric diets with either 14% and 50% energy as total milk protein, and they did see a significant decrease in body weights which is in accordance with the present study. Morens and colleagues (2001) also observed that 50% energy as protein saturated catabolic capacities of metabolising amino acids and was associated with decreased gastric emptying, causing a decrease in intake. However, a three week trial using male rodents consuming 12% and 26% energy as protein with adequate B_6 status showed no differences in weight or intake (Tirapegui et al., 2012).

Typically, higher protein diets tend to result in less weight gain. Protein has a higher thermogenic effect (20-30%) than carbohydrate (5-10%) and fat (0-3%) (Tirapegui et al., 2012). High protein intakes in overweight humans given caloric intakes to provide 0.6 kg/week weight loss over ten weeks using a higher protein diet (30% energy as protein, 40% energy as carbohydrates, 30% energy as lipid) or a normal protein diet (15% energy as protein, 55% energy as carbohydrate, 30% energy as lipid) also showed a slightly higher weight loss with the higher protein group (Layman et al., 2003). Short term (eight weeks) and long term (six months) rodent studies observed decreased body weights, fat mass, retroperitoneal fat pad weight, white adipose

tissue weight and feed intake with higher milk protein consumption (50-53% as energy) compared to low protein intakes (14% as energy), while keeping lipid constant and diets isocaloric (Blouet et al., 2006; Jean et al., 2001; Lacroix et al., 2004). No difference in lean body mass or skeleton weight were also observed in these latter studies indicating increased body lipid as the main reason for increased weights in lower protein diets (14% as energy). Although the present study did not analyze or measure white adipose tissue or retroperitoneal fat pads, a decreased feed intake and body weight was observed. Thus the decreased body weight could result from numerous factors including decreased feed intake, increased utilization of dietary glucose as energy due to suboptimal amino acid metabolism and possible decreased carcass lipid.

The present study's findings of increased ADG in adequate B_6 status consuming 20% energy as protein versus 40% energy as protein is comparable to findings by Morens el al. (2001) who used 14% and 50% energy as total milk protein. Interestingly, a significant interaction between protein and B_6 level for ADG was observed with ADG increasing with 40% energy as protein in moderate B_6 deficiency, while the opposite was seen in the adequate B_6-40% protein group. This is possibly due to increased body water during B_6 deficiency as described by Angel and Song (1973), as increased protein intake doesn't necessary mean increased muscle mass (Garlick, McNurlan, & Patlak, 1999; Lacroix et al., 2004; Tirapegui et al., 2012). Tirapegui et al (2012) found that protein intake at 26% as energy over three weeks did not result in any changes in protein nutritional status as measured by serum albumin, insulin, total protein and insulin-like growth factor 1. According to the review by Garlick, McNurlan & Patlak (1999), there is an acute and long-term response to protein intake. In the acute phase increase amino acid oxidation occurs with increased energy expenditure from this process and an increased amino acid pool retained in the body. After a few days the chronic response sets in and amino acid oxidation

adapts so the body's nitrogen balance approaches close to zero, yet no significant increase in muscle is seen in multiple studies. This is possibly due to a slow incorporation of nitrogen into muscle (Garlick et al., 1999) and no flexible storage capacity for excess amino acid intake resulting in oxidation and elimination (Tirapegui et al., 2012).

Although not significant, the present study observed plasma PLP, PA, PL increasing with adequate B_6 and 40% energy as protein ($p=0.43$, $p=0.47$, $p=0.09$), which has been previously observed (Nguyen & Gregory, 1983). Okada and colleagues observed the opposite effect with rodents consuming 20% or 70% energy as casein and administering equal levels of B_6 for each diet group (Okada et al., 1998a). In comparison to Okada et al. (1998), the present study's adequate B_6-40% protein group observed plasma PLP 1.5 times higher and PL 1.4 times higher than Okada et al (1998), while plasma PLP and PL in the 20% as energy as protein group under the same B_6 conditions was comparable in both studies. This discrepancy in plasma PLP and PL at 40% and 70% energy as protein may reflect a tipping point in the balance of plasma B_6 vitamers in metabolizing protein intake above 40%. The lowering of plasma PLP at 70% energy as protein may indicate a possible increased usage or tissue storage of B_6 at protein intakes above 40%.

Along with increased plasma PLP in adequate B_6-40% protein intake, the present study also observed a significant decrease in plasma PN with 40% energy as protein intake regardless of B_6 status and a significant increase in plasma PL for the interaction of adequate B_6-40% protein intake. This phenomenon can be explained by understanding the interconversion of B_6 vitamers. When the demand for PLP-dependent enzymes increases, so does the requirement for PLP. A review by Merrill Jr. & Henderson (1990) indicates that PL kinase is readily available in many tissues facilitating the phosphorylation of PL, PM, and PN to keep them contained in

tissues. PNP/PMP oxidase however are mostly found in the liver and thus the majority of conversion of PMP and PNP to PLP by this oxidase enzyme is done there (Merrill Jr. & Henderson, 1990). An increased demand for PLP, for reasons such as increased protein intake, causes increased conversion of PMP and PNP to PLP via PNP/PMP oxidase. Non-specific alkaline phosphatase hydrolyses the bond between PL and phosphate to liberate the vitamer from tissues into circulation where PL can be taken up and re-phosphorylated to PLP by the abundant PL kinase enzyme (di Salvo et al., 2011; Merrill Jr. & Henderson, 1990). Merrill Jr & Henderson (1990) indicate the rates of phosphorylating PN, PM and PL are much slower then the conversion of PNP and PMP to PLP. Feed restriction of 60% over forty weeks has been shown to lead to a decrease in B_6 plasma vitamers after ten weeks, increased retention in tissues and an increase in PNP/PMP oxidase enzyme over time (Wei, 1999), indicating increased conversion of other forms of B_6 to PLP. The increased speed of converting PNP and PMP to PLP as well as the abundance of PL kinase available to re-phosphorylate PL is a plausible reason for why we see increased plasma PLP and PL and decreased PN during increased PLP demand. The fact that the short-term indicator of B_6 intake and utilization, PA, was also increased with high protein intake under adequate B_6 status leads to the conclusion that more B_6 was being metabolized and excreted with higher protein intakes and adequate B_6 status.

As expected, marginal B_6 deficiency decreased plasma B_6 vitamers regardless of protein intake except for PA, which stayed the same. Although not significant, 40% energy as protein intake during marginal B_6 deficiency tended to decreased plasma PLP, PL, PN compared to 20% energy as protein, which was the opposite observed in the adequate B_6 group. The decrease in plasma B_6 vitamers is most likely a reflection of tissue retention of the vitamin for vital enzyme functions in animals and humans (Miller et al., 1985; Miyazaki et al., 2011; Moriya et al., 2012;

Pregnolato et al., 1994; Wei, 1999), and a decrease in B_6 utilization and excretion at any protein level as determined by PA and no significant elevations in HCY and CYS.

Although no significant elevations of HCY and CYS were observed in our study a significant decrease in hepatic CGL with marginal B_6 deficiency and also protein intake at 40% was observed. Our previous work shows similar findings, and significant decreases in hepatic CBS and CGL, as in severe B_6 deficiency, have to occur before an elevation in HCY and CYS are observed (Mayengbam et al., 2015). Thus even though the transsulfuration pathway is depressed, there is still enough activity to allow for non-significant elevations in plasma HCY and CYS.

Liver weights in the present study significantly decreased with marginal B_6 deficiency and showed a trend (P=0.06) to decrease with increasing protein intake. There are varying reports on liver weights in conditions of adequate B_6 status and high protein (50%-70% as energy) intake with some studies reporting higher weights (Morens et al., 2001), and some with no change (Jean et al., 2001; Okada et al., 1998a) and others are in agreement with our findings (Pregnolato et al., 1994). Liver weight can be a reflection of total liver lipids, which were not significantly different between groups in the present study, but a decreasing trend (p=0.06) was seen with increased protein intake. The non-significant difference between total liver lipids in marginal and adequate B_6 status was unexpected as it contradicts the findings the author's previous pilot study, which reported an increase in total liver lipids under the same conditions (Raposo, unpublished data). This may be due to small sample size (n=4) of the pilot study, but the exact reasoning is not clear. There is a lack of data on liver lipids in marginal deficiency states, so the results are hard to compare, but work in our lab is observing a more temporal effect

of hepatic lipid accumulation in marginal deficiency, with sensitivity to stage of deficiency (Mayengbam et al., 2015).

Severe B_6 deficiency with 40% and 70% energy as protein does increase liver lipids through impaired lipid metabolism (Pregnolato et al., 1994; Suzuki et al., 1976), so the accumulation of liver lipid may be a consequence of severe B_6 deficiency more so than protein intake. Liver lipid accumulation is also observed with marginal B_6 deficiency tipping over into a severe deficiency as observed when administering 10 mg/kg of 1-Amino-D-Proline during moderate B_6 deficiency (Mayengbam et al., 2015). Decreased total liver lipid may also result from increased substrate utilization as plasma and hepatic TG were significantly decreased in marginal B_6 deficiency. During impairments in endogenous glucose utilization, increased oxidation of fatty acids from TGs are supplemented for the lack of carbon originally intended to come from glucose to enter the TCA cycle (Angel, 1975; Veldhorst et al., 2009). Marginal B_6 deficiency may provide adequate B_6 for PLP-dependent intermediary metabolic enzymes, which allows the utilization of TG for energy and not the trapping of TG in the liver as seen in severe B_6 deficiency (Pregnolato et al., 1994; Suzuki & Okada, 1984). It should be noted that no apparent changes are seen in plasma TG in rodents consuming 14% and 50% energy as protein with adequate B_6 status (Blouet et al., 2006) and administration of glucose during fatty liver induced severe B_6 deficiency reduces hepatic lipids (Suzuki & Okada, 1982b). This helps provide evidence that decreased plasma and hepatic TGs observed in marginal B_6 deficiency are most likely due to the marginal B_6 deficiency and as a result increased demand for carbon skeletons originally supplied by glucose metabolic pathways, which were impaired.

The impairment of the glucose metabolic pathways in marginal B_6 deficiency can be observed in our study by the significant increase in hepatic glycogen, significant decreases in

hepatic glycogen phosphorylase, and gluconeogenic pathways from plasma AST and ALT. These findings are in accordance with other studies observing decreases in these enzymes and other glucose metabolising enzymes (glucokinase and hexokinase) in overt B_6 deficiency (Angel, 1980; Okada & Ochi, 1971; Okada & Hirose, 1979; Toney, 2013). Decreased B_6 status observed in aging male rodents also exhibited a decrease in active glycogen phosphorylase in muscle with only 5% of the enzyme being active (Bode et al., 1991) but the decrease in glycogen phosphorylase in muscle was not as dramatic in female rodents (Bode et al., 1991). Other aging rodent studies indicate that the severe decrease in glycogen phosphorylase activities occurs the most in young growing rats, and the degree of enzyme decrease depends on the severity of PLP depletion in the tissue (Cochary, Gershoff, Sadowski, 1991). These studies indicate that glycogen phosphorylase activity is dependent on PLP content in tissues and is decreased in specific life stages and gender.

Muscle glycogen is mostly used to provide glucose to the muscle itself while liver glycogen is more responsible for maintaining blood glucose levels (Okada, Goda, Murakami, Shibuya, 2000). Okada, et al. (2000) observed decreases in glycogen phosphorylase activity in muscle, heart and liver of severely deficient rodents consuming 70% energy as casein over five weeks, with elevated glycogen contents in muscle and liver, which is concurrent with our study's findings. The authors added PLP to the reaction medium for determination glycogen phosphorylase activity but saw no increased effect on activity, indicating no apoenzymes were present and hypothesized that the apoenzymes for glycogen phosphorylase were degraded because their ability or synthesis of the enzyme is dependent on PLP (Okada et al., 2000). Severe B_6 deficiency has been shown to weaken rodent leg muscles and also decrease glucose utilization by the brain (Wei, Huang, Wang, 1999). Case studies of McArdle Disease in humans, a genetic

glycogenic myopathy where patients exhibit fatigue, reduced exercise tolerance and muscle weakness, oral B_6 supplementation (90 mg PN-HCl/day) has proven successful in increasing residual glycogen phosphorylase activity and improving symptoms (Izumi, et al., 2010; Sato, Takekazu, Nishino, Sugie, 2012). These studies help show the importance for vitamin B_6 in glycogen breakdown via glycogen phosphorylase, which ultimately effects energy utilization. Due to decreases in glycogen phosphorylase during B_6 deficiency, the body is not able to breakdown glycogen to glucose for maintaining blood glucose during fasting and glucose derived carbon skeletons to enter the TCA cycle for energy maintenance. Thus the body must rely on carbon skeletons form stored triglycerides and protein as well as dietary glucose intake to maintain these processes in B_6 deficiency.

No matter what tissue the glycogen is present in, PLP is a cofactor for glycogen phosphorylase and required for the optimal activity of this enzyme and glucose utilization during fasted states. Due to the increased risk of marginal deficiency in the elderly population and the effects of aging on B_6 status and PLP-dependent enzymes, this segment of the population may be at increased risk of altered glucose utilization and metabolic consequences of B_6 even with adequate B_6 intake.

Overt B_6 deficiency and overt deficiency with 70% casein has been observed to decrease plasma AST and ALT (Angel, 1980; Suzuki et al., 1976), but this is the first study, to the authors knowledge, to demonstrate a significant decrease also in marginal B_6 deficiency in a rodent model. This is an important finding as marginal B_6 deficiency is seen in about 18% of the population in North America and up to 50% in Europe (Haller et al., 1991; Morris et al., 2008) and blood AST and ALT are common indications of hepatic injury, but marginal deficiency in B_6 may not accurately reflect hepatic health. Therefore it may be beneficial for clinicians to screen

patients for B_6 status in order to determine if interpretations of blood AST and ALT are accurate or misleading.

Plasma haptoglobin showed no significant differences among the groups, which is concurrent with another study showing no major differences in inflammatory markers with 50% protein intake over six months in adequate B_6 rodents (Lacroix et al., 2004). Only increased central vein dilation was a significant histological indication for increased liver injury with increased protein intake. Yet histological indications of inflammation by observation and grading systems can be objective, which is why quantitative analysis with such methods is key in providing evidence for or against the visual observation. B_6 intake has been associated with significant decreases in molecular expression of inflammatory markers, including tumor necrosis factor (TNF)-α, interleukin (IL)-6 and cyclooxygenase-2 as well as histological markers of inflammation in a mouse model of inflammatory bowel disease (Selhub et al., 2013). PLP has also been shown to serve an important role in genome integrity in small fly cells, as deficiency resulted in increased breaks and re-arrangements in the genome and in the presence of hyperglycemia genetic damage occurred (Marzio, Merigliano, Gatti, Verni, 2014). Although studies have shown B_6 is important for reducing inflammation (Sakakeeny et al., 2012; Yamamoto et al., 2013) and genome integrity (Marzio, Merigliano, Gatti, Verni, 2014), the current study's rodent model of marginal B_6 deficiency may not have had enough time to develop a hepatic insult, and more research into the time to develop these insults is needed.

In summary marginal B_6 deficiency decreases PLP-dependent enzymes in glycolysis, transsulfuration, and transamination. Clinicians should consider taking B_6 status into account before interpreting liver injury markers plasma AST and ALT as these were significantly decreased in marginal deficiency. Intake of 40% energy as protein, close to the upper AMDR

resulted in increased interconversion of PN to PLP, indicating increased demand for PLP during intakes of forty percent energy as protein. Some PLP-dependent enzymes, such as CGL were also negatively affected by 40% energy as protein intake. Therefore the DRI for B_6 should take into consideration B_6 requirement with protein intakes at or above the upper AMDR range, especially for the older adult population, which is more susceptible to lower B_6 status.

8. Strengths and Limitations

The limitations of the study include the discrepancy of body weight change between the present study and the pilot study in marginal B_6 deficiency. Yet all other criteria for meeting marginal deficiency were met (non-significant decrease in plasma HCY and CYS with significant decrease in plasma B_6 vitamers). This discrepancy in body weight may be due to a slightly more deficient model than the pilot study, enough to alter body composition but not plasma biomarkers. The length of time may be a limitation to our study, as liver lipid accumulation or markers of inflammation might change over a longer period in marginal deficiency. This is due to the fact that the consequences for severe deficiency takes affect right away and are visible, while the effect of marginal deficiency are less pronounced and gradual. A long-term study may be more beneficial in determining the long term affects of protein consumption on B_6 status and effects on enzymes intermediary metabolism, whereas our study may be representative of a more acute high protein intake. The other limitation is that we tested for consumption of high intake of casein as the protein source and generalized it as high protein intake. It would be beneficial to look into various forms of protein (plant and animal), to determine how varying proteins effect B_6 status. Fisher, Willis, & Haskell (1984) have observed an average decreased weight gain and B_6 status regardless of vitamin B_6 intake in rodents consuming amino acid mixtures similar to maize (low quality protein) compared to casein. Gender may also be a limitation of the study as only male rodents were used. The age of the rats is also a limitation as we had used growing rats for which the implications of marginal B_6 deficiency may be greater than rats who are in adulthood.

Strengths of this study include findings that indicate the delicate balance between vitamin B_6 and energy utilization and that there are deleterious effects to intermediary metabolising

enzymes occurring in marginal B_6 deficiency. Another strength is that the present study was able to show the novel finding of decreased liver function enzymes (AST and ALT) under marginal B_6 deficiency, which to the writer's knowledge has not been found in the rodent model. Another strength is that we had used a previous established model for moderate vitamin B_6 deficiency, which can allow this study to be compared to others using the same model.

9. Future Research

Gender may be an important variable to investigate as aging rodent studies see differences in PL kinase activity and other B_6 related enzymes in females vs. males (Bode et al., 1991). Future research should include long term and short term comparisons of high protein intakes during marginal B_6 deficiency to determine if there are changes over time. Different protein sources and quality could investigated as casein, soybean and maize have resulted in different results in B_6 status (Fisher, Willis, & Haskell, 1984; Lu & Huang, 1997). With the increasing fad of low carbohydrate diets, another future research study could focus on elevating the protein and lipid content and determining if that diet ratio has consequences to B_6 status.

10. Major findings of Study

Marginal B_6 deficiency:

- Significantly decreases plasma B_6 vitamers and hepatic CGL without significant alterations in HCY and CYS.
- Increases hepatic glycogen and reduces glycogen phosphorylase activity.
- Lowers hepatic and plasma triglycerides.
- Decreases plasma AST and ALT.

Protein intakes at 40% diet energy under marginal B_6 deficiency:

- Further decrease in plasma PN and hepatic CGL.
- Tended to decrease plasma PL ($p=0.09$).

Marginal B_6 deficiency had more detrimental implications to intermediary metabolizing enzymes and B_6 status markers than protein intake at 40% of dietary energy alone (similar to the upper AMDR). Yet significant decreases in plasma PN and a decreasing trend in plasma PL under marginal B_6 deficiency and 40% protein intake suggests increased conversion of those B_6 vitamers in that condition. Therefore the DRI for B_6 should take more notice of protein intakes at or near the upper AMDR when establishing B_6 requirement.

11. References

Abe, M., & Kishino, Y. (1982). Pathogenesis of fatty liver in rats fed a high protein diet without pyridoxine. *Journal of Nutrition, 112*(1), 205-210.

Ahn, H. J., Min, K. W., & Cho, Y. O. (2011). Assessment of vitamin B(6) status in korean patients with newly diagnosed type 2 diabetes. *Nutrition Research and Practice, 5*(1), 34-39.

Allen, G. F., Neergheen, V., Oppenheim, M., Fitzgerald, J. C., Footitt, E., Hyland, K., et al. (2010). Pyridoxal 5'-phosphate deficiency causes a loss of aromatic L-amino acid decarboxylase in patients and human neuroblastoma cells, implications for aromatic L-amino acid decarboxylase and vitamin B(6) deficiency states. *Journal of Neurochemistry, 114*(1), 87-96.

Angel, J. F. (1975). Lipogenesis by hepatic and adipose tissues from meal fed pyridoxine deprived rats. *Nutrition Reports International, 11*(5), 369-378.

Angel, J. F. (1980). Gluconeogenesis in meal-fed, vitamin B-6-deficient rats. *The Journal of Nutrition, 110*(2), 262-269.

Angel, J. F., & Song, G. W. (1973). Lipogenesis in pyridoxine deficient nibbling and meal fed rats. *Nutrition Reports International, 8*(6), 393-403.

Anitha, M., Abraham, P. M., & Paulose, C. S. (2012). Striatal dopamine receptors modulate the expression of insulin receptor, IGF-1 and GLUT-3 in diabetic rats: Effect of pyridoxine treatment. *European Journal of Pharmacology, 696*(1-3), 54-61.

Bauer, J., Biolo, G., Cederholm, T., Cesari, M., Cruz-Jentoft, A. J., Morley, J. E., et al. (2013). Evidence-based recommendations for optimal dietary protein intake in older people: A position paper from the PROT-AGE study group. *Journal of the American Medical Directors Association, 14*(8), 542-559.

Bhagavan, H. N., & Brin, M. (1983). Drug--vitamin B6 interaction. *Current Concepts in Nutrition, 12*, 1-12.

Blouet, C., Mariotti, F., Azzout-Marniche, D., Bos, C., Mathe, V., Tome, D., et al. (2006). The reduced energy intake of rats fed a high-protein low-carbohydrate diet explains the lower fat deposition, but macronutrient substitution accounts for the improved glycemic control. *The Journal of Nutrition, 136*(7), 1849-1854.

Bode, W., Mocking, J. A., & van den Berg, H. (1991). Influence of age and sex on vitamin B-6 vitamer distribution and on vitamin B-6 metabolizing enzymes in wistar rats. *The Journal of Nutrition, 121*(3), 318-329.

Bowling, F. G. (2011). Pyridoxine supply in human development. *Seminars in Cell and Developmental Biology, 22*(6), 611-618.

Cabrini, L., Bochicchio, D., Bordoni, A., Sassi, S., Marchetti, M., & Maranesi, M. (2005). Correlation between dietary polyunsaturated fatty acids and plasma homocysteine concentration in vitamin B6-deficient rats. *Nutrition, Metabolism, and Cardiovascular Diseases : NMCD, 15*(2), 94-99.

Campbell, W. W., Trappe, T. A., Wolfe, R. R., & Evans, W. J. (2001). The recommended dietary allowance for protein may not be adequate for older people to maintain skeletal muscle. *The Journals of Gerontology.Series A, Biological Sciences and Medical Sciences, 56*(6), M373-80.

Cheng, Q., Li, N., Chen, M., Zheng, J., Qian, Z., Wang, X., et al. (2013). Cyclooxygenase-2 promotes hepatocellular apoptosis by interacting with TNF-alpha and IL-6 in the pathogenesis of nonalcoholic steatohepatitis in rats. *Digestive Diseases and Sciences, 58*(10), 2895-2902.

Cho, Y. O., & Leklem, J. E. (1990). In vivo evidence for a vitamin B-6 requirement in carnitine synthesis. *The Journal of Nutrition, 120*(3), 258-265.

Cilliers, K., Labadarios, D., Schaaf, H. S., Willemse, M., Maritz, J. S., Werely, C. J., et al. (2010). Pyridoxal-5-phosphate plasma concentrations in children receiving tuberculosis chemotherapy including isoniazid. *Acta Paediatrica (Oslo, Norway : 1992), 99*(5), 705-710.

Coburn, S. P., Lewis, D. L., Fink, W. J., Mahuren, J. D., Schaltenbrand, W. E., & Costill, D. L. (1988). Human vitamin B-6 pools estimated through muscle biopsies. *The American Journal of Clinical Nutrition, 48*(2), 291-294.

Cochary, E. F., Gershoff, S. N., & Sadowski, J. A. (1991). Effects of vitamin B-6 deficiency and aging on pyridoxal 5'-phosphate levels and glycogen phosphorylase activity in rats. *The Journal of Nutritional Biochemistry, 2*(3), 135-141.

Cohen, J. (1992). A power primer. *Psychological Bulletin, 112*(1), 155-159.

da Silva, V. R., Rios-Avila, L., Lamers, Y., Ralat, M. A., Midttun, O., Quinlivan, E. P., . . . Gregory, J. F.,3rd. (2013). Metabolite profile analysis reveals functional effects of 28-day vitamin B-6 restriction on one-carbon metabolism and tryptophan catabolic pathways in healthy men and women. *The Journal of Nutrition, 143*(11), 1719-1727. doi:10.3945/jn.113.180588 [doi]

Devkota, S., & Layman, D. K. (2011). Increased ratio of dietary carbohydrate to protein shifts the focus of metabolic signaling from skeletal muscle to adipose. *Nutrition and Metabolism, 8*

Di Salvo, M. L., Contestabile, R., & Safo, M. K. (2011). Vitamin B 6 salvage enzymes: Mechanism, structure and regulation. *Biochimica Et Biophysica Acta - Proteins and Proteomics, 1814*(11), 1597-1608.

di Salvo, M. L., Contestabile, R., & Safo, M. K. (2011). Vitamin B(6) salvage enzymes: Mechanism, structure and regulation. *Biochimica Et Biophysica Acta, 1814*(11), 1597-1608.

di Salvo, M. L., Safo, M. K., & Contestabile, R. (2012). Biomedical aspects of pyridoxal 5'-phosphate availability. *Frontiers in Bioscience (Elite Edition), 4*, 897-913.

Dunn, W. A., Aronson, N. N.,Jr, & Englard, S. (1982). The effects of 1-amino-D-proline on the production of carnitine from exogenous protein-bound trimethyllysine by the perfused rat liver. *The Journal of Biological Chemistry, 257*(14), 7948-7951.

Fabian, E., Bogner, M., Kickinger, A., Wagner, K. -., & Elmadfa, I. (2012). Vitamin status in elderly people in relation to the use of nutritional supplements. *Journal of Nutrition, Health and Aging, 16*(3), 206-212.

Feres, D. D., Dos Santos, M. P., Buzelle, S. L., Pereira, M. P., de Franca, S. A., Garofalo, M. A., et al. (2013). In vitro TNF-alpha- and noradrenaline-stimulated lipolysis is impaired in adipocytes from growing rats fed a low-protein, high-carbohydrate diet. *Lipids,*

Fisher, J. H., Willis, R. A., & Haskell, B. E. (1984). Effect of protein quality on vitamin B-6 status in the rat. *The Journal of Nutrition, 114*(4), 786-791.

Folch, J., Lees, M., & Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of Biological Chemistry, 226*(1), 497-509.

Garlick, P. J., McNurlan, M. A., & Patlak, C. S. (1999). Adaptation of protein metabolism in relation to limits to high dietary protein intake. *European Journal of Clinical Nutrition, 53 Suppl 1*, S34-43.

Garriguet, D. (2007). Canadians' eating habits. *Health Reports / Statistics Canada, Canadian Centre for Health Information = Rapports Sur La Sante / Statistique Canada, Centre Canadien D'Information Sur La Sante, 18*(2), 17-32.

Giffen, P. S., Turton, J., Andrews, C. M., Barrett, P., Clarke, C. J., Fung, K. W., et al. (2003). Markers of experimental acute inflammation in the wistar han rat with particular reference to haptoglobin and C-reactive protein. *Archives of Toxicology, 77*(7), 392-402.

Gloria, L., Cravo, M., Camilo, M. E., Resende, M., Cardoso, J. N., Oliveira, A. G., et al. (1997). Nutritional deficiencies in chronic alcoholics: Relation to dietary intake and alcohol consumption. *The American Journal of Gastroenterology, 92*(3), 485-489.

Gregory III, J. F. (1997). Bioavailability of vitamin B-6. *European Journal of Clinical Nutrition, 51*(SUPPL. 1), S43-S48.

Gregory III, J. F., Park, Y., Lamers, Y., Bandyopadhyay, N., Chi, Y. -., Lee, K., et al. (2013). Metabolomic analysis reveals extended metabolic consequences of marginal vitamin B-6 deficiency in healthy human subjects. *PLoS ONE, 8*(6)

Haller, J., Lowik, M. R., Ferry, M., & Ferro-Luzzi, A. (1991). Nutritional status: Blood vitamins A, E, B6, B12, folic acid and carotene. euronut SENECA investigators. *European Journal of Clinical Nutrition, 45 Suppl 3*, 63-82.

Hansen, C. M., Leklem, J. E., & Miller, L. T. (1996). Vitamin B-6 status of women with a constant intake of vitamin B-6 changes with three levels of dietary protein. *The Journal of Nutrition, 126*(7), 1891-1901.

Hansen, C. M., Shultz, T. D., Kwak, H. K., Memon, H. S., & Leklem, J. E. (2001). Assessment of vitamin B-6 status in young women consuming a controlled diet containing four levels of vitamin B-6 provides an estimated average requirement and recommended dietary allowance. *The Journal of Nutrition, 131*(6), 1777-1786.

Hlais, S., Reslan, D. R., Sarieddine, H. K., Nasreddine, L., Taan, G., Azar, S., et al. (2012). Effect of lysine, vitamin B(6), and carnitine supplementation on the lipid profile of male

patients with hypertriglyceridemia: A 12-week, open-label, randomized, placebo-controlled trial. *Clinical Therapeutics, 34*(8), 1674-1682.

Ink, S. L., Gregory III, J. F., & Sartain, D. B. (1986). Determination of pyridoxine ß-glucoside bioavailability using intrinsic and extrinsic labeling in the rat. *Journal of Agricultural and Food Chemistry, 34*(5), 857-862.

Institute of Medicine. (1998). Vitamin B6. *Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenicAcid, biotin, and choline.* (pp. 150). Washington, DC: National Academy Press.

Institute of Medicine. (2005). *
Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids (macronutrients)* . Washington, DC: National Academic Press.

Institute of Medicine. (2006). In Otten J. J., Hellwig J. P. and Meyers L. D. (Eds.), *Dietary reference intakes, the essential guide to nutrient requirements.* Washington, D. C.: The National Academies Press.

Ito, R., & Okada, M. (1973). Effect of dietary protein level on pyridoxal content in tissues and excretion of pyridoxic acid into urine in normal or pyridoxine-deficient rat. *Journal of Nutritional Science and Vitaminology, 19*(6), 523-528.

Izumi, R., Suzuki, N., Kato, K., Warita, H., Tateyama, M., Nakashima, I., & Itoyama, Y. (2010). A case of McArdle disease: Efficacy of vitamin B6 on fatigability and impaired

glycogenolysis. *Internal Medicine (Tokyo, Japan), 49*(15), 1623-1625. doi:JST.JSTAGE/internalmedicine/49.3525 [pii]

Jean, C., Rome, S., Mathe, V., Huneau, J. F., Aattouri, N., Fromentin, G., et al. (2001). Metabolic evidence for adaptation to a high protein diet in rats. *The Journal of Nutrition, 131*(1), 91-98.

Kaimoto, T., Shibuya, M., Nlshlkawa, K., & Maeda, H. (2013). High incidence of lipid deposition in the liver of rats fed a diet supplemented with branched-chain amino acids under vitamin B6 deficiency. *Journal of Nutritional Science and Vitaminology, 59*(1), 73-77.

Keith, M. E., Walsh, N. A., Darling, P. B., Hanninen, S. A., Thirugnanam, S., Leong-Poi, H., et al. (2009). B-vitamin deficiency in hospitalized patients with heart failure. *Journal of the American Dietetic Association, 109*(8), 1406-1410.

Khalesi, S., Irwin, C., & Schubert, M. (2015). Flaxseed consumption may reduce blood pressure: A systematic review and meta-analysis of controlled trials. *The Journal of Nutrition, 145*(4), 758-765.

Kuhnlein, H. V., Receveur, O., Soueida, R., & Berti, P. R. (2008). Unique patterns of dietary adequacy in three cultures of canadian arctic indigenous peoples. *Public Health Nutrition, 11*(4), 349-360.

Lacroix, M., Gaudichon, C., Martin, A., Morens, C., Mathe, V., Tome, D., et al. (2004). A long-term high-protein diet markedly reduces adipose tissue without major side effects in wistar

male rats. *American Journal of Physiology.Regulatory, Integrative and Comparative Physiology, 287*(4), R934-42.

Lamers, Y., O'Rourke, B., Gilbert, L. R., Keeling, C., Matthews, D. E., Stacpoole, P. W., et al. (2009). Vitamin B-6 restriction tends to reduce the red blood cell glutathione synthesis rate without affecting red blood cell or plasma glutathione concentrations in healthy men and women. *American Journal of Clinical Nutrition, 90*(2), 336-343.

Lamers, Y., O'Rourke, B., Gilbert, L. R., Keeling, C., Matthews, D. E., Stacpoole, P. W., et al. (2009). Vitamin B-6 restriction tends to reduce the red blood cell glutathione synthesis rate without affecting red blood cell or plasma glutathione concentrations in healthy men and women. *The American Journal of Clinical Nutrition, 90*(2), 336-343.

Layman, D. K., Shiue, H., Sather, C., Erickson, D. J., & Baum, J. (2003). Increased dietary protein modifies glucose and insulin homeostasis in adult women during weight loss. *The Journal of Nutrition, 133*(2), 405-410.

Leklem, J. E. (1990). Vitamin B-6: A status report. *The Journal of Nutrition, 120 Suppl 11*, 1503-1507.

Lima, C. P., Davis, S. R., Mackey, A. D., Scheer, J. B., Williamson, J., & Gregory, J. F.,3rd. (2006). Vitamin B-6 deficiency suppresses the hepatic transsulfuration pathway but increases glutathione concentration in rats fed AIN-76A or AIN-93G diets. *The Journal of Nutrition, 136*(8), 2141-2147.

Lu, S. -., & Huang, P. -. (1997). Effect of casein and soy protein isolate on vitamin B6 nutritional status in rats. *Journal of Biomedical Science, 4*(2-3), 120-124.

Lui, A., Lumeng, L., Aronoff, G. R., & Li, T. K. (1985). Relationship between body store of vitamin B6 and plasma pyridoxal-P clearance: Metabolic balance studies in humans. *The Journal of Laboratory and Clinical Medicine, 106*(5), 491-497.

Lussana, F., Zighetti, M. L., Bucciarelli, P., Cugno, M., & Cattaneo, M. (2003). Blood levels of homocysteine, folate, vitamin B6 and B12 in women using oral contraceptives compared to non-users. *Thrombosis Research, 112*(1-2), 37-41.

Mackey, A. D., Lieu, S. O., Carman, C., & Gregory III, J. F. (2003). Hydrolytic activity toward pyridoxine-5'-ß-D-glucoside in rat intestinal mucosa is not increased by vitamin B-6 deficiency: Effect of basal diet composition and pyridoxine intake. *Journal of Nutrition, 133*(5), 1362-1367.

Marszałł, M. L., Lebiedzińska, A., Czarnowski, W., Makarowski, R., Kłos, M., & Szefer, P. (2009). Application of the high-performance liquid chromatography method with coulometric detection for determination of vitamin B6 in human plasma and serum. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 877*(27), 3151-3158.

Martinez, M., Cuskelly, G. J., Williamson, J., Toth, J. P., & Gregory, J. F.,3rd. (2000). Vitamin B-6 deficiency in rats reduces hepatic serine hydroxymethyltransferase and cystathionine beta-synthase activities and rates of in vivo protein turnover, homocysteine remethylation and transsulfuration. *The Journal of Nutrition, 130*(5), 1115-1123.

Marzio, A., Merigliano, C., Gatti, M., & Vernì, F. (2014). Sugar and chromosome stability: Clastogenic effects of sugars in vitamin B6-deficient cells. *PLoS Genetics, 10*(3)

Mason, J. K., & Thompson, L. U. (2014). Flaxseed and its lignan and oil components: Can they play a role in reducing the risk of and improving the treatment of breast cancer? *Applied Physiology, Nutrition, and Metabolism = Physiologie Appliquee, Nutrition Et Metabolisme, 39*(6), 663-678.

Masse, P. G., Boudreau, J., Tranchant, C. C., Ouellette, R., & Ericson, K. L. (2012). Type 1 diabetes impairs vitamin B(6) metabolism at an early stage of women's adulthoods. *Applied Physiology, Nutrition, and Metabolism = Physiologie Appliquee, Nutrition Et Metabolisme,*

Mayengbam, S., Raposo, S., Aliani, M., & House, J. D. (2015). Oral exposure to the anti-pyridoxine compound 1-amino d-proline further perturbs homocysteine metabolism through the transsulfuration pathway in moderately vitamin B6 deficient rats. *The Journal of Nutritional Biochemistry, 26*(3), 241-249.

Mayengbam, S., Yang, H., Barthet, V., Aliani, M., & House, J. D. (2014). Identification, characterization, and quantification of an anti-pyridoxine factor from flaxseed using ultrahigh-performance liquid chromatography-mass spectrometry. *Journal of Agricultural and Food Chemistry, 62*(2), 419-426.

McArthur, J. O., Tang, H., Petocz, P., & Samman, S. (2013). Biological variability and impact of oral contraceptives on vitamins B6, B12 and folate status in women of reproductive age. *Nutrients, 5*(9), 3634-3645.

Mehta, R., Dedina, L., & O'Brien, P. J. (2011). Rescuing hepatocytes from iron-catalyzed oxidative stress using vitamins B1 and B6. *Toxicology in Vitro : An International Journal Published in Association with BIBRA, 25*(5), 1114-1122.

Merrill Jr., A. H., & Henderson, J. M. (1990). Vitamin B6 metabolism by human liver. *Annals of the New York Academy of Sciences, 585*, 110-117.

Midttun, Ø, Hustad, S., Schneede, J., Vollset, S. E., & Ueland, P. M. (2007). Plasma vitamin B-6 forms and their relation to transsulfuration metabolites in a large, population-based study. *American Journal of Clinical Nutrition, 86*(1), 131-138.

Miller, J. W., Nadeau, M. R., Smith, D., & Selhub, J. (1994). Vitamin B-6 deficiency vs folate deficiency: Comparison of responses to methionine loading in rats. *The American Journal of Clinical Nutrition, 59*(5), 1033-1039.

Miller, L. T., Leklem, J. E., & Shultz, T. D. (1985). The effect of dietary protein on the metabolism of vitamin B-6 in humans. *The Journal of Nutrition, 115*(12), 1663-1672.

Miyazaki, A., Sano, M., Fukuwatari, T., & Shibata, K. (2011). Effects of ethanol consumption on the B-group vitamin contents of liver, blood and urine in rats. *The British Journal of Nutrition,* , 1-8.

Morens, C., Gaudichon, C., Fromentin, G., Marsset-Baglieri, A., Bensaid, A., Larue-Achagiotis, C., et al. (2001). Daily delivery of dietary nitrogen to the periphery is stable in rats adapted to increased protein intake. *American Journal of Physiology.Endocrinology and Metabolism, 281*(4), E826-36.

Moriya, A., Fukuwatari, T., Sano, M., & Shibata, K. (2012). Different variations of tissue B-group vitamin concentrations in short-and long-term starved rats. *British Journal of Nutrition, 107*(1), 52-60.

Morris, M. S., Picciano, M. F., Jacques, P. F., & Selhub, J. (2008). Plasma pyridoxal 5'-phosphate in the US population: The national health and nutrition examination survey, 2003-2004. *The American Journal of Clinical Nutrition, 87*(5), 1446-1454.

Nguyen, L. B., & Gregory, J. F.,3rd. (1983). Effects of food composition on the bioavailability of vitamin B-6 in the rat. *The Journal of Nutrition, 113*(8), 1550-1560.

Okada, M., Goda, H., Kondo, Y., Murakami, Y., & Shibuya, M. (2001). Effect of exercise on the metabolism of vitamin B6 and some PLP-dependent enzymes in young rats fed a restricted vitamin B6 diet. *Journal of Nutritional Science and Vitaminology, 47*(2), 116-121.

Okada, M., & Hirose, M. (1979). Regulation of aspartate aminotransferase activity associated with change of pyridoxal phosphate level. *Archives of Biochemistry and Biophysics, 193*(1), 294-300.

Okada, M., Ishikawa, K., & Watanabe, K. (1991). Effect of vitamin B6 deficiency on glycogen metabolism in the skeletal muscle, heart, and liver of rats. *Journal of Nutritional Science and Vitaminology, 37*(4), 349-357.

Okada, M., & Ochi, A. (1971). The effect of dietary protein level on transaminase activities and fat deposition in vitamin B 6 -depleted rat liver. *Journal of Biochemistry, 70*(4), 581-585.

Okada, M., Shibuya, M., Akazawa, T., Muya, H., & Murakami, Y. (1998a). Dietary protein as a factor affecting vitamin B6 requirement. *Journal of Nutritional Science and Vitaminology, 44*(1), 37-45.

Okada, M., Shibuya, M., Akazawa, T., Muya, H., & Murakami, Y. (1998b). Dietary protein as a factor affecting vitamin B6 requirement. *Journal of Nutritional Science and Vitaminology, 44*(1), 37-45.

Okada, M., Shibuya, M., Yamamoto, E., & Murakami, Y. (1999). Effect of diabetes on vitamin B6 requirement in experimental animals. *Diabetes, Obesity & Metabolism, 1*(4), 221-225.

O'Leary, F., Flood, V. M., Petocz, P., Allman-Farinelli, M., & Samman, S. (2011). B vitamin status, dietary intake and length of stay in a sample of elderly rehabilitation patients. *The Journal of Nutrition, Health & Aging, 15*(6), 485-489.

Ollilainen, V. (1999). HPLC analysis of vitamin B6 in foods. *Agricultural and Food Science in Finland, 8*(6), 526-618.

Pannemans, D. L., van den Berg, H., & Westerterp, K. R. (1994). The influence of protein intake on vitamin B-6 metabolism differs in young and elderly humans. *The Journal of Nutrition, 124*(8), 1207-1214.

Pazirandeh, S., Lo, C. W., & Burns, D. (2009, Up To Date). Overview of water soluble vitamins. Message posted to www.uptodate.com

Percudani, R., & Peracchi, A. (2003). A genomic overview of pyridoxal-phosphate-dependent enzymes. *EMBO Reports, 4*(9), 850-854.

Pfeiffer, C. M., Sternberg, M. R., Schleicher, R. L., Haynes, B. M., Rybak, M. E., & Pirkle, J. L. (2013). The CDC's second national report on biochemical indicators of diet and nutrition in the U.S. population is a valuable tool for researchers and policy makers. *The Journal of Nutrition, 143*(6), 938S-47S.

Pregnolato, P., Maranesi, M., Marchetti, M., Barzanti, V., Bergami, R., & Tolomelli, B. (1994). Interaction among dietary vitamin B6, proteins and lipids: Effects on liver lipids in rats. *International Journal for Vitamin and Nutrition Research, 64*(4), 263-269.

Raposo, S. (2012). *Pyridoxine deficiency and homocysteine: Their effects on liver lipids.* Unpublished manuscript.

Reeves, P. G., Nielsen, F. H., & Fahey, G. C.,Jr. (1993). AIN-93 purified diets for laboratory rodents: Final report of the american institute of nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *The Journal of Nutrition, 123*(11), 1939-1951.

Rios-Avila, L., Coats, B., Chi, Y. Y., Midttun, O., Ueland, P. M., Stacpoole, P. W., & Gregory, J. F.,3rd. (2015). Metabolite profile analysis reveals association of vitamin B-6 with metabolites related to one-carbon metabolism and tryptophan catabolism but not with biomarkers of inflammation in oral contraceptive users and reveals the effects of oral contraceptives on these processes. *The Journal of Nutrition, 145*(1), 87-95. doi:10.3945/jn.114.201095 [doi]

Rose, C. S., Gyorgy, P., Butler, M., Andres, R., Norris, A. H., Shock, N. W., et al. (1976). Age differences in vitamin B6 status of 617 men. *The American Journal of Clinical Nutrition, 29*(8), 847-853.

Saarinen, N. M., Smeds, A. I., Penalvo, J. L., Nurmi, T., Adlercreutz, H., & Makela, S. (2010). Flaxseed ingestion alters ratio of enterolactone enantiomers in human serum. *Journal of Nutrition and Metabolism, 2010*, 10.1155/2010/403076. Epub 2010 May 5.

Said, H. M. (2011). Intestinal absorption of water-soluble vitamins in health and disease. *The Biochemical Journal, 437*(3), 357-372.

Sakakeeny, L., Roubenoff, R., Obin, M., Fontes, J. D., Benjamin, E. J., Bujanover, Y., et al. (2012). Plasma pyridoxal-5-phosphate is inversely associated with systemic markers of inflammation in a population of U.S. adults. *Journal of Nutrition, 142*(7), 1280-1285.

Sato, S., Ohi, T., Nishino, I., & Sugie, H. (2012). Confirmation of the efficacy of vitamin B6 supplementation for McArdle disease by follow-up muscle biopsy. *Muscle & Nerve, 45*(3), 436-440. doi:10.1002/mus.22290 [doi

Scheer, J. B., Mackey, A. D., & Gregory, J. F.,3rd. (2005). Activities of hepatic cytosolic and mitochondrial forms of serine hydroxymethyltransferase and hepatic glycine concentration are affected by vitamin B-6 intake in rats. *The Journal of Nutrition, 135*(2), 233-238.

Selhub, J., Byun, A., Liu, Z., Mason, J. B., Bronson, R. T., & Crott, J. W. (2013). Dietary vitamin B intake modulates colonic inflammation in the IL10 model of inflammatory bowel disease. *The Journal of Nutritional Biochemistry,*

Shakur, Y. A., Tarasuk, V., Corey, P., & O'Connor, D. L. (2012). A comparison of micronutrient inadequacy and risk of high micronutrient intakes among vitamin and mineral supplement users and nonusers in canada. *The Journal of Nutrition, 142*(3), 534-540.

Simpson, K. J., Lukacs, N. W., Colletti, L., Strieter, R. M., & Kunkel, S. L. (1997). Cytokines and the liver. *Journal of Hepatology, 27*(6), 1120-1132.

Steel, D. M., & Whitehead, A. S. (1994). The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein. *Trends in Immunology, 15*(2), 81-88.

Suzuki, K., Nakamura, T., Fujita, M., Iwami, T., & Abe, M. (1976). Factors affecting liver lipid content in pyridoxing-deficient rats. I. dietary protein levels. *Journal of Nutritional Science and Vitaminology, 22*(4), 291-298.

Suzuki, K., & Okada, M. (1982a). Alterations of phospholipid and triglyceride metabolism in fatty liver caused by pyridoxine deficiency in rats. *Journal of Nutritional Science and Vitaminology, 28*(4), 377-390.

Suzuki, K., & Okada, M. (1982b). Role of glucose on fatty liver formation in pyridoxine-deficient rats. *Journal of Nutritional Science and Vitaminology, 28*(4), 367-375.

Suzuki, K., & Okada, M. (1984). Studies on the origin of triglyceride in fatty liver caused by pyridoxine-deficiency in rats. *Nutrition Research, 4*(4), 691-699.

Talwar, D., Quasim, T., McMillan, D. C., Kinsella, J., Williamson, C., & O'Reilly, D. S. (2003). Optimisation and validation of a sensitive high-performance liquid chromatography assay for routine measurement of pyridoxal 5-phosphate in human plasma and red cells using pre-column semicarbazide derivatisation. *Journal of Chromatography.B, Analytical Technologies in the Biomedical and Life Sciences, 792*(2), 333-343.

Tanaka, N., Matsubara, T., Krausz, K. W., Patterson, A. D., & Gonzalez, F. J. (2012). Disruption of phospholipid and bile acid homeostasis in mice with nonalcoholic steatohepatitis. *Hepatology (Baltimore, Md.), 56*(1), 118-129.

Taysi, S., Keles, M. S., Gumustekin, K., Akyuz, M., Boyuk, A., Cikman, O., & Bakan, N. (2015). Plasma homocysteine and liver tissue S-adenosylmethionine, S-adenosylhomocysteine status in vitamin B6-deficient rats. *European Review for Medical and Pharmacological Sciences, 19*(1), 154-160. doi:8350 [pii]

Tirapegui, J., Ribeiro, S. M. L., Pires, I. S. O., & Rogero, M. M. (2012). Effects of two different levels of dietary protein on body composition and protein nutritional status of growing rats. *Nutrients, 4*(9), 1328-1337.

Toney, M. D. (2013). Aspartate aminotransferase: An old dog teaches new tricks. *Archives of Biochemistry and Biophysics,*

Ubbink, J. B., Serfontein, W. J., & de Villiers, L. S. (1985). Stability of pyridoxal-5-phosphate semicarbazone: Applications in plasma vitamin B6 analysis and population surveys of vitamin B6 nutritional status. *Journal of Chromatography, 342*(2), 277-284.

Ulvik, A., Theofylaktopoulou, D., Midttun, Ø, Nygard, O., Eussen, S. J. P. M., & Ueland, P. M. (2013). Substrate product ratios of enzymes in the kynurenine pathway measured in plasma as indicators of functional vitamin B-6 status. *American Journal of Clinical Nutrition, 98*(4), 934-940.

Vaz, F. M., & Wanders, R. J. (2002). Carnitine biosynthesis in mammals. *The Biochemical Journal, 361*(Pt 3), 417-429.

Veldhorst, M. A. B., Westerterp-Plantenga, M. S., & Westerterp, K. R. (2009). Gluconeogenesis and energy expenditure after a high-protein, carbohydrate-free diet. *American Journal of Clinical Nutrition, 90*(3), 519-526.

Viñas, P., Balsalobre, N., López-Erroz, C., & Hernández-Córdoba, M. (2004). Determination of vitamin B6 compounds in foods using liquid chromatography with post-column derivatization fluorescence detection. *Chromatographia, 59*(5-6), 381-386.

Wakefield, A. P., House, J. D., Ogborn, M. R., Weiler, H. A., & Aukema, H. M. (2011). A diet with 35% of energy from protein leads to kidney damage in female sprague-dawley rats. *British Journal of Nutrition, 106*(5), 656-663.

Wei, I., (1999). The influence of dietary restriction on vitamin B-6 vitamer distribution and on vitamin B-6 metabolizing enzymes in rats. *Journal of the American College of Nutrition, 18*(2), 144-151.

Wei, I. L., Huang, Y. H., & Wang, G. S. (1999). Vitamin B6 deficiency decreases the glucose utilization in cognitive brain structures of rats. *The Journal of Nutritional Biochemistry, 10*(9), 525-531. doi:S0955-2863(99)00040

Whitehouse, C. R., Boullata, J., & McCauley, L. A. (2008). The potential toxicity of artificial sweeteners. *AAOHN Journal : Official Journal of the American Association of Occupational Health Nurses, 56*(6), 251-9; quiz 260-1.

Whitehouse, L. W., Tryphonas, L., & Paul, C. J. (1983). Isoniazid-induced hepatic steatosis in rabbits: An explanation for susceptibility and its antagonism by pyridoxine hydrochloride. *Canadian Journal of Physiology and Pharmacology, 61*(5), 478-487.

Wrenger, C., Muller, I. B., Schifferdecker, A. J., Jain, R., Jordanova, R., & Groves, M. R. (2011). Specific inhibition of the aspartate aminotransferase of plasmodium falciparum. *Journal of Molecular Biology, 405*(4), 956-971.

Xiao, Y., Zhang, Y., Lv, X., Su, D., Li, D., Xia, M., et al. (2011). Relationship between lipid profiles and plasma total homocysteine, cysteine and the risk of coronary artery disease in coronary angiographic subjects. *Lipids in Health and Disease, 10*, 137.

Yamamoto, K., Isa, Y., Nakagawa, T., & Hayakawa, T. (2013). Involvement of 5-methyltetrahydrofolate in the amelioration of hyperhomocysteinemia caused by vitamin B(6) deficiency and L-methionine supplementation. *Bioscience, Biotechnology, and Biochemistry, 77*(2), 378-380.

Yang, S. Q., Lin, H. Z., Yin, M., Albrecht, J. H., & Diehl, A. M. (1998). Effects of chronic ethanol consumption on cytokine regulation of liver regeneration. *The American Journal of Physiology, 275*(4 Pt 1), G696-704.

10. Appendix

Pearson Correlation

	Plasma PN	Plasma PLP	Plasma PA	Plasma PL	Total ADI	Final weight	Total ADG	Relative liver weight	Hepatic CGL	Hepatic TG	Plasma TG	Hepatic glycogen	Plasma ALT	Plasma AST	Hepatic GP Activity
Plasma PN	.391*	.523**	.426*	.606**	.605**	.520**	.606**	.536**	.443*	.292	-.467**	.318	.296	.392*	
Plasma PLP	.716**	.964**	.682**	.499**	.565**	.499**	.712**	.682**	.376*	-.506**	.789**	.750**	.584**		
Plasma PA	.667**	.538**	.483*	.448*	.482*	.533**	.589**	.410	-.107	.553**	.665**	.597**			
Plasma PL	.638**	.558**	.600**	.558**	.675**	.733**	.428*	-.529**	.748**	.723**	.523**				
Total ADI	.794**	.765**	.795**	.819**	.689**	.391*	-.621**	.615**	.374*	.630**					
Final weight	.868**	1.000**	.644**	.596**	.529**	-.575**	.367*	.331	.498**						
Total ADG	.868**	.698**	.454*	.554**	-.656**	.513**	.272	.423*							
Relative liver weight	.645**	.596**	.531**	-.575**	.368*	.332	.498**								
Hepatic CGL	.606**	.449*	-.425*	.759**	.525**	.613**									
Hepatic TG	.413*	-.379*	.597**	.546**	.418*										
Plasma TG	-.261	.414*	.431*	.347											

	Hepatic glycogen	-.447*	-.176	-.222
		Plasma ALT	.510**	.305
			Plasma AST	.446*
				Hepatic GP Activity

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Summary of Studies on Protein and B_6 Intake on Hepatic Lipids

Title, Author, Year	Description & Methods	Results
Role of Glucose on Fatty Liver Formation in PN-Deficient Rats, Suzuki & Okada, (1982)-	Does glucose (glc) starvation= fatty liver in B_6- rats? Rats fed 70 then 20 % casein. Pair fed. 0 mg PN vs. unknown adequate amount.	No sig diff plasma glc conc. but 43.9%↓ in liver. ↓ liver glycogen. ↓ active insulin=↓ glc uptake. ↓ Glukokinase & hexokinase. B_6- & ↑ protein =↑ liver fat. Going from 70 to 20 % protein casein sig ↓ liver lipids. Liver lipids tended to ↓ after glc injection.
Factors affecting liver lipid content in pyridoxine-deficient rats. Dietary protein levels, Suzuki, et al., (1976)	Looked @ liver lipid content vs dietary protein. Trial 1= protein vs. liver lipid 10, 30, 50 70% casein. Trial 2= liver lipid vs. asp-t activity 70% casein. Trial 3= fatty liver vs. time 70% casein. Trial 4= diet change vs. liver lipid 20 & 70 % casein. Pair fed groups.0 mg PN vs. unknown adequate amount.	B_6- = relative liver enlargement in 50-70% casein but ↓ in 10-30 % casein vs controls. Critical point observ. b/w 30-50 % casein related to liver growth in B_6-. ASP-AT ↓ w/↑ protein. Liver lipid close related to level of liver ASP-AT activity. 70% casein=↑ liver lipid after 1wk, 2.3 times ↑ vs. controls @ wk 3. ↓lipids when casein ↓ or when supplemented with PN, but liver still enlarged.
Pathogenesis of Fatty Liver in Rats Fed a High Protein Diet Without PN, Abe, et al. (1982)	Do hepatocytes (HPC) change structure in development of fatty liver in rats fed high protein diet and B_6-? Rats fed 70% casein & B_6-. Pair fed controls. 0 mg PN vs. unknown adequate amount. Killed at 3 D, 1, 2, 4, 8, 16 wks. PN given after 8 wks and recovery examined @ 1, 2, 3 weeks.	Relative liver wt ↑ in B_6- & 40% ↑ liver lipids, lipid droplets in hepatocytes ↑ w/time,↑ TG conc. @ 2 wks droplets centered in hepatocyte becoming widespread w/time. Droplet size varied, larger in center, seen in cytoplasm. @ 16 wks droplets=few & smaller. Foamy cells w/small nuclei observed. No necrosis or fibrosis seen. When PN added lipid droplets in HPC disappeared after 3 wks. Control group=no lipids in HPC or abnormalities. When sufficient PN given, most abnormalities reversed, except for myelin figures in hepatocytes.
Interaction Among Dietary Vitamin B_6, Proteins and Lipids: Effects on Liver Lipids in rats, Pregnolato, et al., (1994)	B_6 relationship & lipid metabolism. Looked at B_6- vs. ↑ protein & low EFA diet. Rats fed combo of B_6- (0.02 mg PN) & B_6+ (4 mg PN), 20 & 40% protein, low EFA & normal lipid diet. Not pair fed.	Level of protein affected body wt. B_6- = ↓ body wt & liver wt. ↑ protein diet = general ↑ fat in liver. Diet composition causes some diff in FA & total lipid composition. B_6-= alterations in lipid metabolism.
Isoniazid-Induced Hepatic Steatosis in Rabbits: an Explanation for Susceptibility and its Antagonism by Pyridoxine Hydrochloride, Whitehouse, et al., (1982)	Does B_6 reduce/prevents isoniazid induced fatty liver? Rabbits administered w/isoniazid alone and in combination with PN-HCl. Gave rabbits 25 mg PN/ kg bodyweight.	Hepatic fatty degeneration observ, lipid droplets found evenly in cytoplasm. Droplet size relates to severity of degeneration. B_6 w/ isoniazid= liver fat developed ↓ rate. Plasma lipid concen. Tended to ↑ more slowly in B_6 w/isoniazid. B_6 helps inhibit these symptoms caused by isoniazid.
The Influence of Dietary Restriction on Vitamin B-6 Vitamer Distribution and on Vitamin B-6 Metabolizing	How does diet restriction (DR) influence B_6 metabolism, tissue concen. & B_6 metabolizing enzymes? 60% restriction in diet for 10, 20 &40 weeks. 0.7 g PN-HCl.	A ↓ body wt & food efficiency ratio. With age a relative ↓ in B_6 intake seen in both groups. DR groups had sig ↓ in PL kinase and PMP(PNP) oxidase activities than controls @ all times. After 20 wks DR group had 29% lower plasma PLP.

Enzymes in Rats, Wei (1999)		
The Effect of Dietary Protein Level on Transaminase Activities and Fat Deposition in Vitamin B_6-Depleted Rat Liver, Okada & Ochi, (1971)	Diff levels of protein in diet vs. transaminase activities in B6- & looked at fatty liver induced by B_6-. Rats pair fed, 10, 20, 70 % casein. B_6 described as deficient or not.	Transaminase activities ↑ up to 7 fold in 70 % B_6+ casein group vs 10 % casein. B_6- 70% casein=↓activity & group liver lipids ~ double B_6+ 70% group. 10% casein B_6- & B_6+ = same liver lipid levels. Observed fatty degeneration of liver cells.
Studies on the Origin of Triglyceride in Fatty Liver Caused by Pyridoxine Deficiency in Rats, Suzuki (1984)	Mechanisms of lipids ↑ in liver, their transport, & origin of TG in liver in B_6-. Rats pair fed, 70% casein & B_6- (0 mg PN) or unknown adequate amount. Another group pair fed, B_6- & given radioactive linoleic acid.	Plasma tubidity test in B_6- = ↓ fat in blood & ↑ into liver vs. controls. ↑ TG, ↓ microsomal TG transfer protein, serum VLDL, Apo-C, & % B-lipoprotein.
Alterations of Phospholipid and Triglyceride Metabolism in Fatty Liver Caused by Pyridoxine Deficiency in Rats, Suzuki & Okada, (1982)	Looked at B_6- on liver TG and Phospholipid metabolism. Rats were pair fed 70% casein. 0 mg PN vs. unknown adequate amount. Radioactive FA used to determine FA metabolism.	B_6- = ↓ tissue fat vs. ↑ liver fat. Relative liver enlargement. ↑ total liver fat, TG, cholesterol, cholesterol ester. No difference in serum TG & free FA, ↓ serum cholesterol & ester & phospholipid. ↑ TG incorporated into liver, as TG ↑ Phospholipids ↓. TG/phospholipid ratio not due to ↓ liver TG hydrolysis or ↑ flow of free FA from tissues to liver.
Dietary Protein as a Factor affecting Vitamin B_6 Requirement, Okada et al., (1998)	Fed rats 20 or 70% casein w/different amounts of B_6 to see B_6 requirement with varying protein intake. Exp 1=Each group was fed a 20% casein diet with 0mg/kg (Group 20-0), 1.45mg/kg (Group 20-0.5), 2.9mg/kg (Group 20-1) or 5.8mg/kg (Group 20-2) PN-HCl. EXP 2=24male weanling rats were divided into 4 groups as well. Each group was fed a 70% casein diet containing 0mg/kg (Group 70-0), 2.9mg/kg (Group 70-1), 5.8mg/kg (70-2) or 8.7mg/kg (70-3) pyridoxine hydrochloride . 4 weeks	Growth sig ↓ in in severe B6- (0 mg B_6 groups) but more in the 70-0 group. Liver wt's didn't differ but increased in group 70-3. Plasma PLP, PL ↑as B_6 intake ↑. 70% protein groups had more suppressed plasma B_6 vitamers vs. 20% protein @ intakes of at the same B_6 dose. There is different B_6 tissue saturation at 20 & 70% intakes. Group 20-1 and 70-2 had saturated B_6 levels.
Lipogenesis by Hepatic and Adipose Tissues from Meal-Fed Pyridoxine-Deprived Rats, Angel (1975)	Looked at B_6- on lipogenesis in liver and adipose. Meal fed 0 mg vs 22 mg PN-HCl/kg to rats over 8 weeks with pair fed controls. Used to 14C-labeled alanine or glutamate as tracers for gluconeogenesis in vivo.	both groups incorporated glucose carbon into FA and glyceride glycerol= lipogenic capacity was not altered. B_6- = small but sig ↓ in lipid in peripheral adipose tissue. B_6- = sig decrease in glc-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, malic enzyme and acetyl CoA carboxylase in both liver and adipose tissue (these are energy producing NADPH-generating enzymes).

Abbreviations: Sig=significant, diff=difference, wt=weight, TG=triglyceride, vs.=versus, wk=week, @=at, concen=concentration, B_6- = B_6 deficiency, glc=glucose.